# LOCALISATION OF ANTIOXIDANTS AND OXIDATIVE MARKERS WITHIN THE ATHEROSCLEROTIC PLAQUE

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# Abstract

Atherosclerosis is a complex inflammatory disease in which oxidative stress is a major protagonist in the development and progression of the atherosclerotic plaque. All biochemical analysis studies of plaque over the past fifteen years have been carried out on whole plaque with no attempt to localise sites of differing biochemical conditions. This study set out to identify in oxidation levels and inflammatory markers in relation to spatial localisation within the plaque.

Advanced plaque samples removed during endarectomy were obtained from the Christchurch Hospital Department of surgery and were dissected into 3-5 mm sections along the longitudinal axis prior to analysis. Samples were analysed for vitamin E, neopterin, total cholesterol and markers of oxidative damage to protein and lipids. Neopterin is a marker of inflammation as it is released by activated macrophages yet it has never been measured in plaques. Initial analysis showed that the acid precipitation method for removing protein from samples prior to HPLC neopterin analysis was causing a significant loss in neopterin. A new acetonitrile based protein removal procedure was developed.

Markers of oxidative stress and inflammation where shown to vary across the length of an atherosclerotic plaque. This variation allows for localized incidences of high and low radical flux and microenvironments of depleted antioxidants or areas in which the prooxidative actions of molecular components are favoured. Significant correlations were rarely seen in more then one plaque and trends found in the combined data set generally did not hold true in individual plaques. This reflects upon the complexity of the disease, especially at this advanced stage in which the biochemical morphology of individual plaques is extremely diverse. Separation of the plaques into pre-, post-, and bifurcation areas did produce some trends. These can be related to shear stress variations in the blood flow; further investigations into the biochemical differences between these areas may provide a better understanding of the growth and development of the atherosclerotic plaque.

# Abbreviations

| | |
|---|---|
| 78NP | 7,8-Dihydroneopterin |
| α-Toc$^{\bullet}$ | α-Tocopheroxyl radical |
| α-TocH | α-Tocopherol |
| ACN | Acetonitrile |
| ANOVA | Analysis of variance |
| apoB100 | Apolipoprotein B100 |
| ATP | Adenosine triphosphate |
| BCA | Bicinchoninic Acid |
| BHT | Butylated hydroxytoluene |
| BSA | Bovine serum albumin |
| $Cu^{+}$ | Cuprous ion |
| $Cu^{2+}$ | Cupric ion |
| CV% | Coefficient of variation |
| DNA | Deoxyribonucleic acid |
| DNPH | 2,4-Dinitrophenyl hydrazine |
| DOPA | 3,4-Dihydroxyphenylalanine |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| ESS | Endothelial shear stress |
| $Fe^{2+}$ | Ferrous ion |
| $Fe^{3+}$ | Ferric ion |
| GSH | Glutathione |
| GTP | Glutathione triphosphate |
| $H^{+}$ | Hydrogen ion |
| $H_2O_2$ | Hydrogen peroxide |
| HCl | Hydrochloric acid |
| HIV | Human immunodeficiency virus |
| HO$^{\bullet}$ | Hydroxyl radical |
| HOCl | Hypochlorous acid |
| HPLC | High performance liquid chromatography |
| $I_2$ | Iodine |

| | |
|---|---|
| iNOS | Inducible nitric oxide synthase |
| KI | Potassium iodide |
| $L^{\bullet}$ | Lipid radical |
| LDL | Low density lipoprotein |
| LH | Lipid |
| $LO^{\bullet}$ | Lipid alkoxyl radical |
| $LOO^{\bullet}$ | Lipid peroxyl radical |
| LOOH | Lipid hydroperoxide |
| LS mean | Least square of the mean |
| MDA | Malondialdehyde |
| MPO | Myeloperoxidase |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| $O_2$ | Molecular oxygen |
| $O_2^{-\bullet}$ | Superoxide |
| oxLDL | Oxidised low density lipoprotein |
| PB-DOPA | Protein bound-3,4-dihydroxyphenylalanine |
| PrH | Protein |
| PrOOH | Protein hydroperoxide |
| PUFAs | Polyunsaturated fatty acid |
| $R^{\bullet}$ | Free radical |
| ROS | Reactive oxygen species |
| R-SH | Reduced thiol |
| SD | Standard deviation |
| SE | Standard error |
| TBA | 2-Thiobarbituric acid |
| TBARS | Thiobarbituric acid reactive substances |
| TCA | Trichloroacetic acid |
| TFA | Trifluoroaceitc acid |
| TMP | Tocopherol mediated peroxidation |
| TNF- α | Tumor necrosis factor-α |
| TPMET | Transplasma membrane electron transport |
| Tyr | Tyrosine |
| TIA | Transient ischemic attack |

# Introduction

## Atherosclerosis

Atherosclerosis is the leading cause of morbidity and mortality in western society. Statistics New Zealand lists cardiovascular disease as accounting for more then 40% of deaths for 2000. Atherosclerosis has only recently been widely acknowledged as an inflammatory disease (Libby et al. 2002), and progresses throughout the lifetime of the individual. The early stages of the disease have been found to be present in infants (Pesonen et al. 1990), although clinical manifestations usually only present themselves in individuals older then fifty years (Milne et al. 2003). While the exact causes and mechanisms of the disease are yet to be elucidated several “risk factors” have been identified. These include age, gender, obesity, cigarette smoking, hypertension, diabetes mellitus, and hyperlipidemia (Stocker and Keaney Jr 2004).

Atherosclerosis is a chronic condition characterised by the migration of monocytes into the intima of arteries. These differentiate into macrophages which become lipid loaded by the uptake of low density lipoproteins. Proliferation and death of these cells contributes to a gradual hardening of the arteries and a narrowing of the vessel lumen that impedes the blood flow. Over time the atherosclerotic lesion can become unstable and suddenly rupture causing formation of a thrombus which can block blood supply to vital organs such as the heart and brain. The result of which is heart attack and stroke, respectively, and these can be collectively termed as cardiovascular disease.

The research described in this thesis has looked at the balance between oxidative stress and antioxidant activity within the atherosclerotic plaque which drive plaque development. Specifically the research involved the examination of plaques removed from patients arteries and determined the concentration of a range of specific markers for various known cell oxidative stress processes.

### Plaque Development and Morphology

The normal arterial wall is composed of three defined layers; the tunica intima, the tunica media and the arterial adventia (Saladin 2007). The innermost layer is the

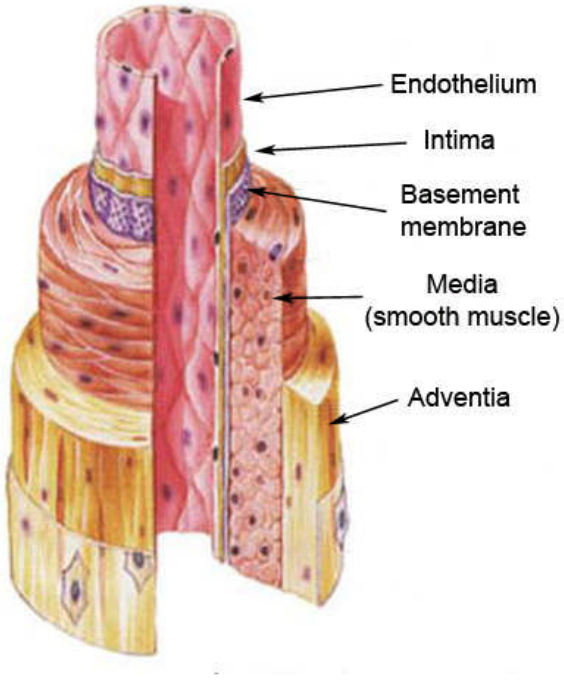


**Figure 1: Composition of the normal arterial wall.**

Adapted from (Fox 1993)

tunica intima and this is composed of three further layers; the endothelium, the intima and the basement membrane (Figure 1).

The endothelial cells of the endothelium act as a physical and functional blockade from the flowing blood. Endothelial cells also play a regulatory role in many arterial processes including vascular tone and blood pressure regulation, absorption of materials and leukocyte trafficking (Silverthorn 2004). These actions are achieved in part through the numerous mechanoreceptors on the cell surface which respond to endothelial shear stress stimuli (Chatzizisis et al. 2007). This induces a signaling cascade which leads to the activation of transcription factors that ultimately modulates cell function and morphology (Chatzizisis et al. 2007).

The intima is normally comprised of a thin layer of loose connective tissue between the endothelium and basement membrane (Saladin 2007). The basement membrane is a layer of elastic tissue. Surrounding the tunica intima is the tunica media. The media consists of layers of smooth muscle cells interspersed with an extracellular matrix composed predominantly of elastin and collagen. The outermost layer of the artery is the arterial adventia and is composed of connective tissue in a loose matrix of elastin, smooth muscle cells, fibroblasts and collagen (Saladin 2007).

## Lesion Initiation

The first observable stage of atherogenesis is commonly termed as a “fatty streak” due to the sub-endothelial lipid deposition which is visible to the naked eye. Lesions of this type can be commonly observed in children and by the third decade of life can occupy as much as one-third of the surface of the aorta on autopsy examination

(Stary et al. 1995). However, the lesions are found only in certain specific locations of the aorta.

The area of incidence for atherosclerotic lesions correlates strongly with locations in which the blood experiences disturbed flow through the arteries such as at arterial branch points and curvatures (Davies 2000). These areas are subjected to a localised incidence of oscillating and low endothelial shear-stress which can produce different functional characteristics in the endothelial cells of these regions (Chatzizisis et al. 2007). These include increased production of leukocyte adhesion molecules, increased NADPH oxidase activity and reduced nitric oxide synthesis; which can contribute to chronic inflammation (Libby et al. 2002). Low endothelial shear stress also promotes the uptake, synthesis and permeability of low density lipoprotein (LDL) (Chatzizisis et al. 2007). Increased synthesis is due to the sustained activation of endothelial transcription factors, caused by low shear stress, which up regulate LDL gene expression (Liu et al. 2002). The transcription factors also promote the accumulation of monocytes via induction of interleukin-8 (Yeh et al. 2004). The disrupted shear stress may also promote arterial smooth muscle cells to produce lipoprotein binding proteoglycans which could contribute to the increased uptake and retention of LDL in the intima of these areas (Libby et al. 2002).

The characteristic feature of fatty streaks is the presence of lipid filled macrophages termed foam cells. Various hypotheses have been proposed to account for the recruitment of monocytes and subsequent lipid loading of the differentiated macrophages. The three major hypotheses have been termed as the response-to-injury; the response-to-retention; and oxidative modification.

The response-to-injury hypothesis proposes that endothelium denudation initiates atherogenesis leading to monocyte and platelet recruitment to the site of injury. These release cytokines, vasoactive agents and growth factors which initiate inflammation. Macrophages are recruited to the area as part of this inflammatory response where they accumulate LDL to form foam cells and perpetuate the inflammation (Jang et al. 1993). Recently, however, it has been shown that denudation of the endothelium is not common and thus the theory has been refined proposing that endothelial dysfunction is capable of initiating the atherogenic process through increased permeability to atherogenic lipoproteins (Stocker and Keaney Jr 2004).

The response-to-retention hypothesis suggests that lipoprotein retention, rather than endothelial permeability, is the initiating factor in atherogenesis. The accumulation of LDL is theorised to be a result of apolipoprotein B-100 motifs that mediate proteoglycan binding and arterial factors which aid lipoprotein aggregation (Williams and Tabas 1995). Aggregated LDL is readily internalised by macrophages and would thus facilitate foam cell formation.

The oxidative modification hypothesis suggests that LDL itself is not atherogenic until it has been modified. LDL that is caught in the subendothelial region can undergo cell-mediated oxidation by the resident smooth muscle cells, endothelial cells and by macrophages (Stocker and Keaney Jr 2004). Oxidised LDL (oxLDL) stimulates monocyte chemotaxis and has been demonstrated to be taken up by macrophages in an unregulated fashion via the scavenger receptor (Goldstein et al. 1979) thus supporting foam cell formation.

The hypotheses outlined above represent the different theories as to what the necessary initial events that generate the atherosclerotic lesion are. They should not be viewed as mutually exclusive and undoubtedly each plays a part in lesion development. The key events in lesion development and the early stages of progression are illustrated in Figure 2.

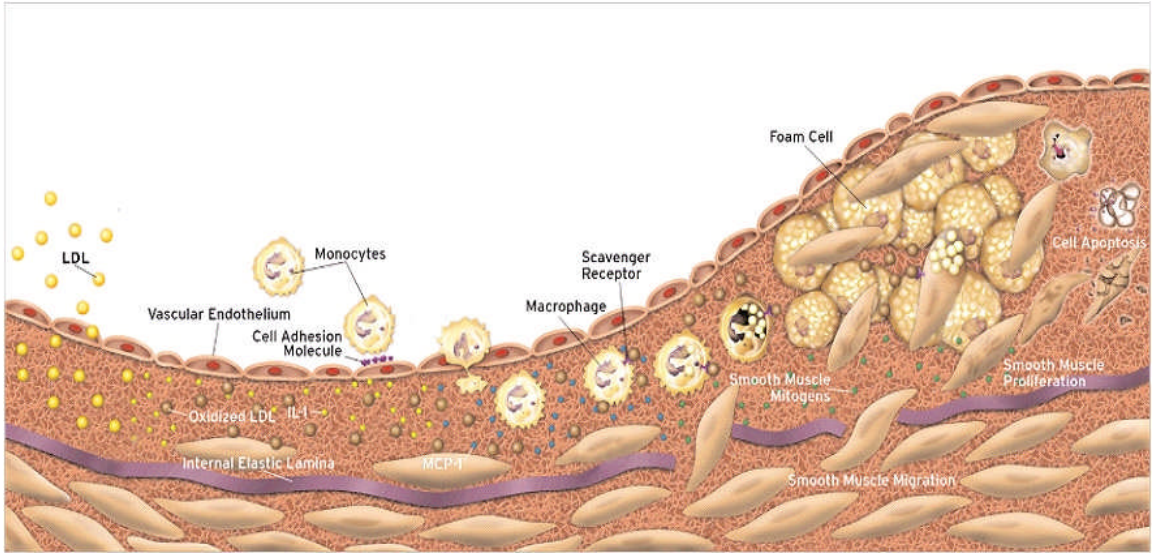


**Figure 2: Evolution of an atherosclerotic plaque.**
Adapted from (Libby 2001).

**Lesion Progression**

Whatever the initiating events, lesion progression is marked by migration and proliferation of smooth muscle cells, the development of an acellular lipid pool which is covered by a fibrous cap and mineral (calcium) deposition.

Continued cytokine release by foam cells, macrophages and T-cells along with the release of growth factors by activated platelets leads to the proliferation and migration of smooth muscle cells in the intima. Under normal conditions smooth muscle cells serve to regulate wall tension; however, under these atherogenic conditions, smooth muscle cells develop a synthetic phenotype (Jang et al. 1993). This change is accompanied by further release of self-stimulating growth factors by the smooth muscle cells themselves. This results in a gradual growth of the plaque over time.

Throughout the atherogenic process macrophages continue to be recruited into the plaque and differentiate to the foam cell morphology. Foam cell death results in the release of the accumulated lipid leading to the formation of an acellular lipid core. In early lesions the cells undergo apoptotic death and a cleared by the normal phagocytotic pathways (Harada et al. 1997, Tabas 2005). In more advanced stages of atherosclerosis oxidative damage causes phagocytosis to be defective and the apoptosis of macrophages leads to secondary necrosis and a pro-inflammatory response (Hegyi et al. 1996, Tabas 2005). This was demonstrated by the presence of extracellular apoptotic fragments in the lipid core (Hegyi et al. 1996). Additionally, some of the extracellular lipid appears to be derived directly from plasma derived lipoproteins aggregating in the extracellular matrix (Stary et al. 1995).

The lipid core is usually covered by a thickened “cap” region composed of fibrotic material. This reparative connective tissue forms in the regions of the intima around the lipid core where the normal cell and intercellular matrix has been disrupted. This extracellular matrix consists predominantly of collagen interspersed with smooth muscle cells (Stary et al. 1995).

Another feature in some advanced atherosclerotic plaques is mineral deposition in the form of calcium hydroxyapatite. Mineral deposits in atherosclerotic plaques have been associated with elastic fibres and it has been suggested that vesicles in the extracellular matrix derived from dead cells may serve as sites for calcification (Stary et al. 1995). It has also been demonstrated that cholesterol and calcium phosphates can accumulate together in lesions as crystal agglomerates (Laird et al. 2006). Alternatively,

it has been suggested that this vascular calcification closely resembles the process of osteogenesis, or bone formation, which involves the specific deposition of calcium by cells (Abedin et al. 2004, Danilevicius et al. 2007).

**Plaque Rupture, Thrombosis and Vascular Remodeling**

Obstruction of arteries and gradual loss of blood flow due to growth of plaques was once thought to be the main cause of clinical events; however, it is now clear that rupture of the plaque leading to thrombis formation is the primary event leading to acute vascular events such as myocardial infarction or stroke.

The integrity of the arterial wall is maintained by the synthesis and breakdown of matrix proteins, mediated primarily by vascular smooth muscle cells and matrix metalloproteinases (Galis and Khatri 2002). The fibrous tissues of the plaque supply the structural integrity whereas the lipid core is weak and highly thrombogenic. Unsurprisingly then, rupture risk factors include a thin fibrous cap and large pools of lipid. Plaque rupture tends to occur at sights shown to have high circumferential stress which is often at the shoulders of the fibrous caps (Arroyo and Lee 1999).

The shoulder areas also show a high level of infiltration by T-cells and macrophages. Activated T-cells produce the cytokine interferon-γ which destabilises the plaque by preventing proliferation of smooth muscle cells and decreasing synthesis of collagen fibrils (Van Der Wal and Becker 1999). Interferon-γ also activates macrophages and thus stimulates the release of matrix metalloproteinases which degrade the extracellular matrix and further destabilises the plaque (Arroyo and Lee 1999).

The degree of disturbance to the plaque which leads to thrombus formation can differ substantially; from small surface erosions to deep tearing of the intima extending into the lipid atheroma (Van Der Wal and Becker 1999). Depending upon the size of the thrombus it can either be incorporated into the lesion, contributing to the rapid progression of the plaque, or it can lead to an acute vascular event (stroke or .

The progression and stability of an atherosclerotic plaque is also determined in part by the vascular remodeling brought about in response to the atherosclerosis (Nakamura et al. 2001). This remodeling can take the form of expansive (outward) remodeling or constrictive (inward) remodeling (Chatzizisis et al. 2007) (Figure 3).

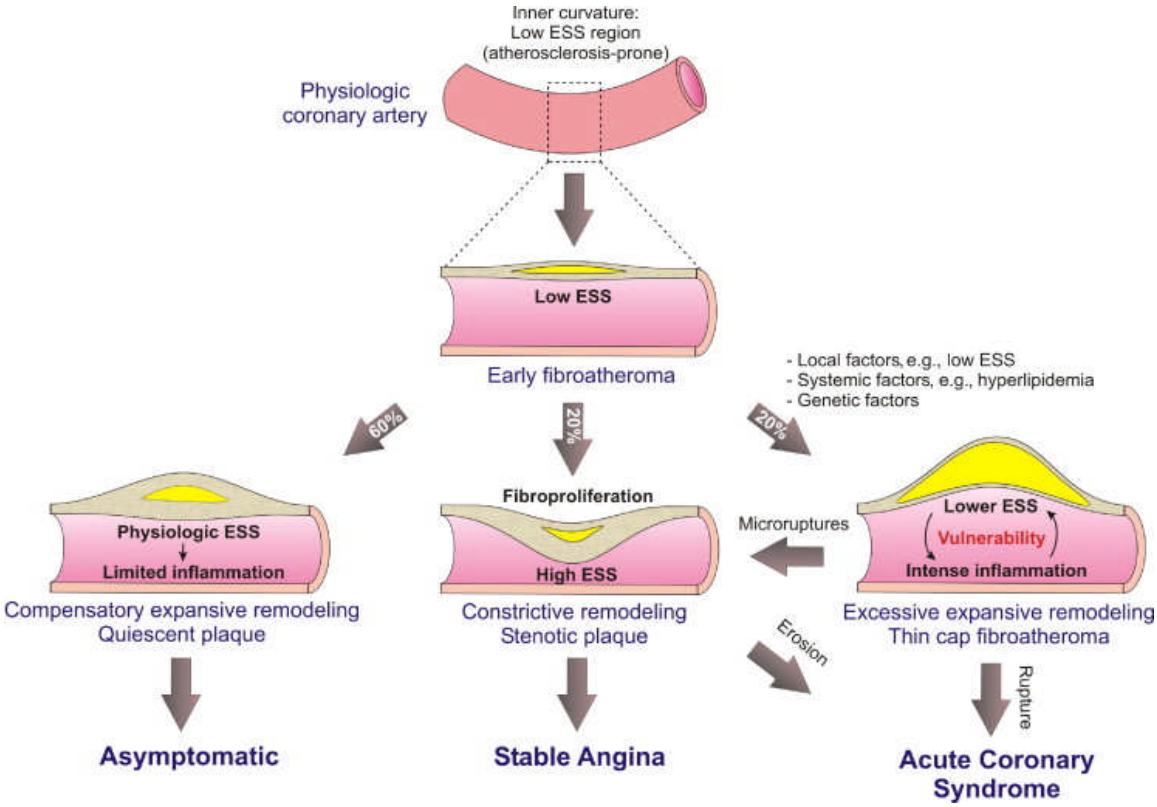


**Figure 3: Vascular remodeling in atherosclerosis.**
Adapted from (Chatzizisis et al. 2007)

Expansive remodeling is a process of arterial enlargement in response to atherosclerosis that allows the lumen to remain preserved allowing the local endothelial shear stress (ESS) to become normalised, reducing inflammation and leading to a quiescent, asymptomatic plaque (Chatzizisis et al. 2007). However in some cases (~20%) excessive expansive remodeling occurs to produce a region in which both the vessel and the lumen are larger then normal neighboring areas (Sipahi et al. 2006). In these areas low shear stress persists, promoting further plaque progression and vessel expansion and subjecting the arterial wall to intense inflammation which leads to a thinly capped fibroatheroma which is prone to rupture (Chatzizisis et al. 2007). Constrictive remodeling occurs when fibroproliferative processes dominate leading to narrowing of the lumen and is thought to be a direct result of plaque growth (Chatzizisis et al. 2007). Constrictive remodeling may also develop through the process of wound healing in response to micro-ruptures in high-risk plaques (Burke et al. 2001).

## Plaque Classification

The composition of advanced atherosclerotic lesions and their precursors have been placed into histological classification by the American Heart Association (Stary et al. 1995) and are summarized below. Many of these types can be found in different zones across the length of an advanced atherosclerotic plaque.

Type I are the initial lesions consisting of isolated macrophage foam cells. Type II lesions are fatty streaks and consist predominantly of intracellular lipid accumulation. Types I and II are sometimes collectively termed as early lesions and are generally present from the first decade of life.

Type III lesions are alternatively referred to as intermediate lesions and consist of small extracellular lipid pools. Type IV lesions, or atheromas, are the first stage of advanced lesions and histologically show a core of extracellular lipid. Type IV lesions are generally present from the third decade of life on.

Type V lesions have extensive thickening of the intima by reparative fibrous tissue layers, and are separated into three subclassifications: Va (fibroatheroma) has the presence of fibrous connective tissue layers along with one or more lipid core; Vb (calcific lesion) is a fibrous lesion which has been significantly calcified; and Vc (fibrotic lesion) is a fibrous lesion which has little or no lipid core and little or no calcification.

Type VI, or complicated, lesions are characterised by some form of surface defect, hematoma or thrombus superimposed upon the existing plaque histology.

Lesion type's I-III are clinically silent and do not narrow the lumen or obstruct the blood flow. Type IV lesions only obstruct blood flow minimally and generally manifest no clinical symptoms. Type V lesions can either be clinically silent or overt depending on the degree of artery obstruction and type VI lesions usually cause significant obstruction of the arteries and are often symptomatic.

# Oxidative Stress within the Atherosclerotic Plaque

Oxidative damage is considered to be a key factor in the initiation and progression of atherosclerosis (Libby et al. 2002). Elevated levels of oxidised lipids, proteins and sterols have been detected in all stages of human atherosclerotic plaques

showing that oxidative events have occurred or are occurring within the plaque (Upston et al. 2002). Oxidation of LDL is considered to be a crucial component of its unregulated uptake and accumulation by macrophages. It is also toxic to cells thus contributing to formation of the lipid core and decreasing plaque stability (Baird et al. 2005, Reid and Mitchinson 1993).

Classical oxidised LDL (oxLDL) is formed through Fenton-type reactions with reduced-metal ions and by cell-mediated oxidation (Figure 4) (Esterbauer et al. 1992). The oxidation of LDL creates multiple atherogenic properties which are unobserved in native LDL in addition to its uncontrolled uptake by macrophages. OxLDL is cytotoxic to most cells and chemotactic for monocytes. It increases the expression of monocyte chemotactic proteins on vascular cells, stimulates expression of vascular cell adhesion molecules and induces macrophage proliferation. OxLDL is also inhibits cholesterol export from macrophages, interferes with endothelium dependent relaxation of blood vessels and is prone to aggregation (Esterbauer et al. 1992, Jessup et al. 2004).

Elevated levels of reactive oxygen species (ROS) and oxLDL can also contribute to the formation of the acellular lipid core by triggering the death of macrophage foam cells. The sources of oxidative stress within the plaque include cellular oxidants, enzymatic oxidants, reduced metal ions and radicals generated on lipids and proteins from initial oxidative events (Figure 4).

The predominant source of ROS is considered to be from the production of superoxide ($O_2^{-\bullet}$) by macrophages. Superoxide dismutases to hydrogen peroxide (reaction 1) which then reacts with reduced transition metals via the fenton reaction to produce the highly reactive hydroxyl radical (reaction 2):

(1) $$2O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$$

(2) $$H_2O_2 + Fe^{2+}/Cu^+ \rightarrow Fe^{3+}/Cu^{2+} + OH^- + {}^{\bullet}OH$$

Atherosclerotic plaques have considerable amounts of catalytic iron and copper present to facilitate this reaction (Minqin et al. 2005, Smith et al. 1992, Stadler et al. 2004). Reduced iron can also catalyse the peroxidation of lipids directly via a fenton-type reaction thus further contributing to the atherogenic environment (Fuhrman et al. 1994, Gieseg and Esterbauer 1994, Murr et al. 1994).

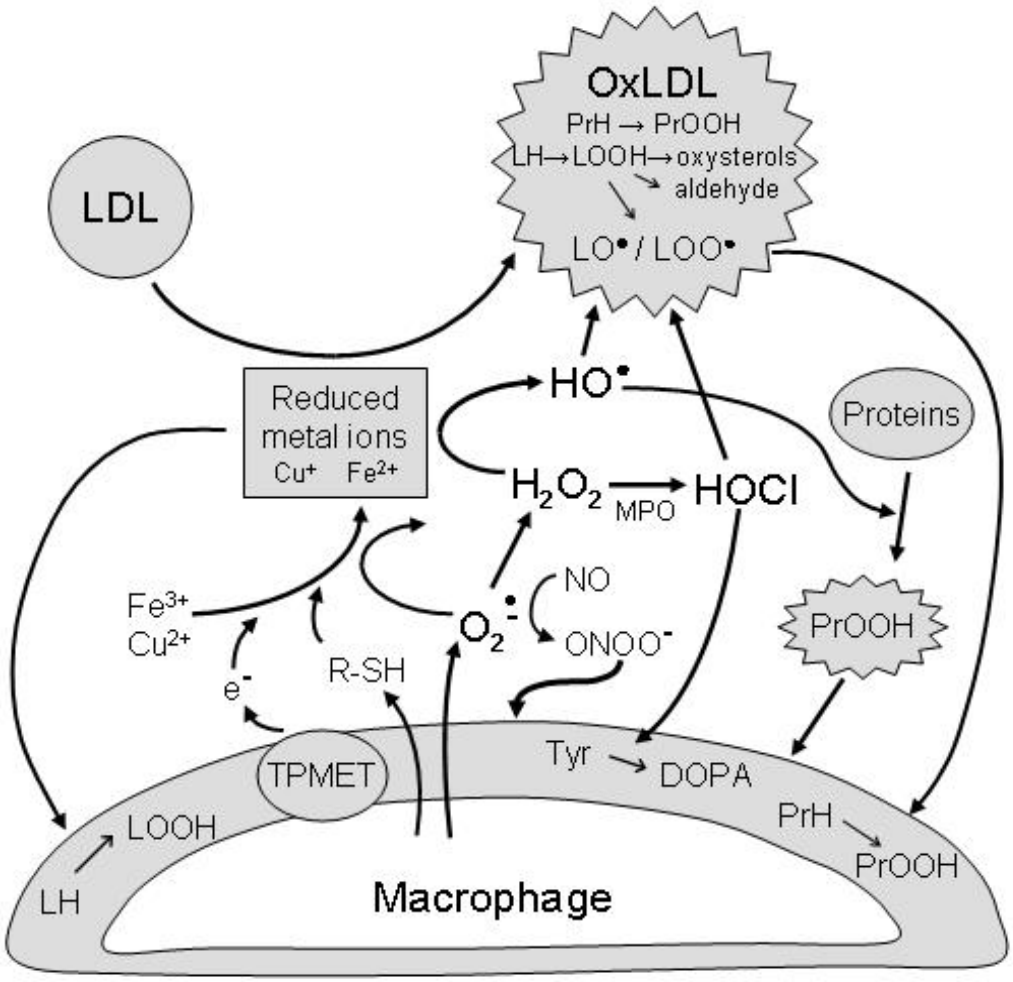


**Figure 4: Oxidant generation within an atherosclerotic plaque.**

Oxidants can be generated through the release of superoxide ($O_2^{-\bullet}$) which can dismutase to hydrogen peroxide ($H_2O_2$). $H_2O_2$ can either form the hydroxyl radical ($HO^\bullet$) via the fenton reaction using reduced metal ions or it can be converted to hypochlorous acid (HOCl) by myeloperoxidase (MPO). $HO^\bullet$ can directly attack the cell, oxidise low density lipoprotein (LDL) or oxidise proteins to protein hydroperoxide (PrOOH). HOCl can oxidise both lipid and proteins in LDL to form oxLDL or it can react with the cell plasma membrane. One of the products of HOCl damage is the oxidation of tyrosine (Tyr) to DOPA which occurs on protein amino acid residues. During oxidation of LDL to oxLDL proteins (PrH) and lipids (LH) are oxidised to protein hydroperoxides (PrOOH) and lipid peroxides (LOOH). Lipid peroxyl ($LOO^\bullet$) and lipid alkoxyl ($LO^\bullet$) radicals can cause further lipid and protein oxidation. Macrophages potentially increase the pool of reduced metal ions through the release of reduced thiols (R-SH) or direct electron transfer via the plasma membrane electron transport complex (TPMET).

Along with the formation of hydroxyl radicals hydrogen peroxide can alternatively react with chloride ions to form hypochlorous acid (HOCl) when aided by the enzyme myeloperoxidase (MPO). This heme protein is secreted by activated phagocytes during inflammation (Heinecke 2002). Active MPO has been shown to present within the atherosclerotic lesion (Daugherty et al. 1994) and high levels of MPO are associated strongly with coronary artery disease (Zhang et al. 2001).

Macrophages further contribute to the oxidative environment of the plaque through the release of reductants. The transplasma membrane electron transport system (TPMET) of macrophages transfers electrons to external ferric and cupric ions enabling participation in the fenton reaction (Baoutina et al. 2001). This recycling of metal ions to their reduced forms is further enhanced by reduced thiol release by macrophages (Graham et al. 1994).

The polyunsaturated fatty acids (PUFAs; LH), which are a large component of LDL and cell membranes, are readily oxidised by radicals ($R^{\bullet}$) (reaction 3) to give a lipid radical ($L^{\bullet}$) which immediately reacts with molecular oxygen (reaction 4). This lipid peroxidation is particularly damaging as it creates a self-perpetuating chain-reaction through creation of the lipid peroxyl radical ($LOO^{\bullet}$) which can oxidise neighboring PUFAs (reaction 5). The resultant lipid hydroperoxides (LOOH) can break down to produce further radicals and a range of other compounds including aldehydes (reaction 6) which can diffuse from the original site thus spreading the damage (Cheeseman and Slater 1993). The presence of oxidised lipids in all stages of atherosclerotic plaques have been reported on widely (Carpenter et al. 1995, Suarna et al. 1995, Yia-Herttuala et al. 1989)

(3) $$LH + R^{\bullet} \rightarrow L^{\bullet} + RH$$

(4) $$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$$

(5) $$LOO^{\bullet} + L'H \rightarrow LOOH + L'^{\bullet}$$

(6) $$LOOH \rightarrow LO^{\bullet}, LOO^{\bullet}, \text{aldehydes}$$

Proteins are also vulnerable to oxidation. Protein bound-DOPA (PB-DOPA), dityrosine, protein carbonyls, protein hydroperoxides and protein bound-chlorotyrosine have all been identified in plaques (Fu et al. 1998, Upston et al. 2002, Woods et al.

2003). The action of hydroxyl radicals and hypochlorous acid on protein tyrosine residues produces PB-DOPA (Gieseg et al. 1993, Sutherland et al. 2003). PB-DOPA is able to perpetuate oxidative stress by reducing copper and iron ions making them available to participate in the fenton reaction and thus catalysing the production of radicals (Morin et al. 1998).

Protein hydroperoxides are the primary product generated from the attack of hydroxyl radicals on proteins (Simpson et al. 1992). In LDL the formation of protein hydroperoxides on the apoB100 protein moiety depends upon the formation of the peroxyl radical in the lipid phase of the LDL molecule (Gieseg et al. 2003). Protein hydroperoxides can cause considerable disruption to normal cell functions as they are able to deactivate enzymes and have been demonstrated to cross-link with DNA (Gebicki 1997). They are also capable of depleting antioxidant pools through the oxidation of glutathione (GSH) and ascorbate (Gebicki 1997, Kappler et al. 2007, Simpson et al. 1992). Upon breakdown protein hydroperoxides form protein carbonyls which are a relatively unreactive end-product that is normally eliminated by proteolysis (Davies et al. 1999).

## Antioxidants within the Atherosclerotic Plaque

Given that oxidative stress plays a pivotal role in the formation of the atherosclerotic plaque it is equally important to examine antioxidant content. The antioxidant enzymes; glutathione peroxidase, glutathione reductase and superoxide dismutase and the non-proteinaceous antioxidants; vitamin C, uric acid, vitamin E and ubiquinol-10 have all been measured within atherosclerotic plaques (Stocker and Keaney Jr 2004). The presence of these antioxidants does not preclude oxidative damage. Temporal and spatial variation in concentrations of antioxidants may allow the sustained oxidative stress to induce damage.

### Vitamin E and Ascorbate

The lipid soluble antioxidant α-tocopherol, which is the most biologically active isoform of vitamin E, is localised in membranes and lipoproteins and is the major

antioxidant present in LDL. Given the importance of LDL oxidation to plaque formation; the content and antioxidant activity of α-tocopherol within atherosclerotic lesions is of considerable interest.

α-Tocopherol (α-TocH) reacts rapidly with lipid peroxyl radicals and interrupts the chain reaction of lipid peroxidation (reaction 7). This forms a relatively stable tocopheroxyl radical (α-Toc$^{\bullet}$) which will normally react with a second radical species to produce stable non-radical products (reaction 8). However, in cases of low radical flux, the tocopheroxyl radical can react with PUFAs by extracting a hydrogen to regenerate tocopherol and produce a new lipid radical (reaction 9). This effect is referred to as tocopherol mediated peroxidation (TMP) and only occurs when there are no lipid peroxyl radicals for the tocopheroxyl radical to react with. This is because of the comparatively slower reaction rate between the tocopheroxyl radical and unoxidised lipids (Bowry and Stocker 1993). The new lipid peroxyl radical generated can consequentially initiate a new autoxidation chain reaction.

(7) $$\alpha\text{-TocH} + LOO^{\bullet} \rightarrow \alpha\text{-Toc}^{\bullet} + LOOH$$

(8) $$\alpha\text{-Toc}^{\bullet} + LOO^{\bullet} \rightarrow \underset{\text{tocopherlylquinone}}{\text{unreactive}} + LOOH \quad (10^6 - 10^7)$$

(9) $$\alpha\text{-Toc}^{\bullet} + LH \rightarrow \alpha\text{-TocH} + L^{\bullet} \quad (100 - 10^4)$$

Even in cases of low radical flux TMP can be protected against through reaction with co-antioxidants such as ascorbate and ubiquinol-10 which can transfer the radical to the aqueous phase (Bowry and Stocker 1993). Ascorbate does this directly across the phase boundary, whereas the lipophilic ubiquinol-10 radical is assumed to react with molecular oxygen to give water soluble superoxide (Bowry and Stocker 1993). The detection of these co-antioxidants along with α-tocopherol in atherosclerotic lesions (Suarna et al. 1995) may restrict the relevance of TMP *in vivo*.

The radical scavenger ascorbate is one of the major intra- and extra-cellular water-soluble antioxidants. Along with radical scavenging and it role as a co-antioxidant to vitamin E, ascorbate can also break down protein hydroperoxides to unreactive hydroxylated amino acid residues (Simpson et al. 1992). Ascorbate can also inhibit copper- and cell-mediated oxidation of LDL which could potentially lower the quantity of oxLDL in plaques (Esterbauer et al. 1989, Retsky et al. 1993, Retsky and Frei 1995, Stait and Leake 1994). However, ascorbate also has the ability to reduce metal ions which can be a source of oxidative stress within the plaque environment (Stait and Leake 1994).

## 7,8-Dihydroneopterin and Neopterin

7,8-Dihydroneopterin (78NP) is released from primate macrophages following stimulation by the cytokine γ-interferon (Gieseg et al. 2007, Muller et al. 1991). γ-Interferon upregulates the action of GTP-cyclohydrolase which catalyses the conversion of GTP to 7,8-dihydroneopterin triphosphate. Primate macrophages contain low levels of the enzyme 6-pyruvoyltetrahydropterin synthase which is the next enzymatic step towards 5,6,7,8-tetrahydrobiopterin. Instead the 7,8-dihydroneopterin triphosphate is converted to 78NP by intracellular phosphotases and diffuses out of the cells (Schoedon et al. 1987) (Figure 5).

Neopterin is the highly fluorescent compound produced by the oxidation of 78NP by hypohalous acids (Gieseg et al. 2000, Widner et al. 2000) (Figure 5) and is used as a marker of inflammation and the immune response (Huber et al. 1983). The level of neopterin in biological fluids is measured either by ELISA or by HPLC with fluorescence detection. Though the initial purchase price of a HPLC is relatively high compared to ELISA equipment, the cost in consumables for each individual sample is relatively inexpensive making it the method of choice in many research laboratories. Neopterin measurement by HPLC is discussed in more detail in the results and discussion of this thesis.

Measurement of plasma and urine neopterin have been used in the clinical management of numerous diseases including HIV infection (Fuchs et al. 1989), autoimmune diseases (Reibnegger et al. 1986), bacterial infections (Strohmaier et al. 1996) and post-operative transplant patients (Margreiter et al. 1983). In relation to heart

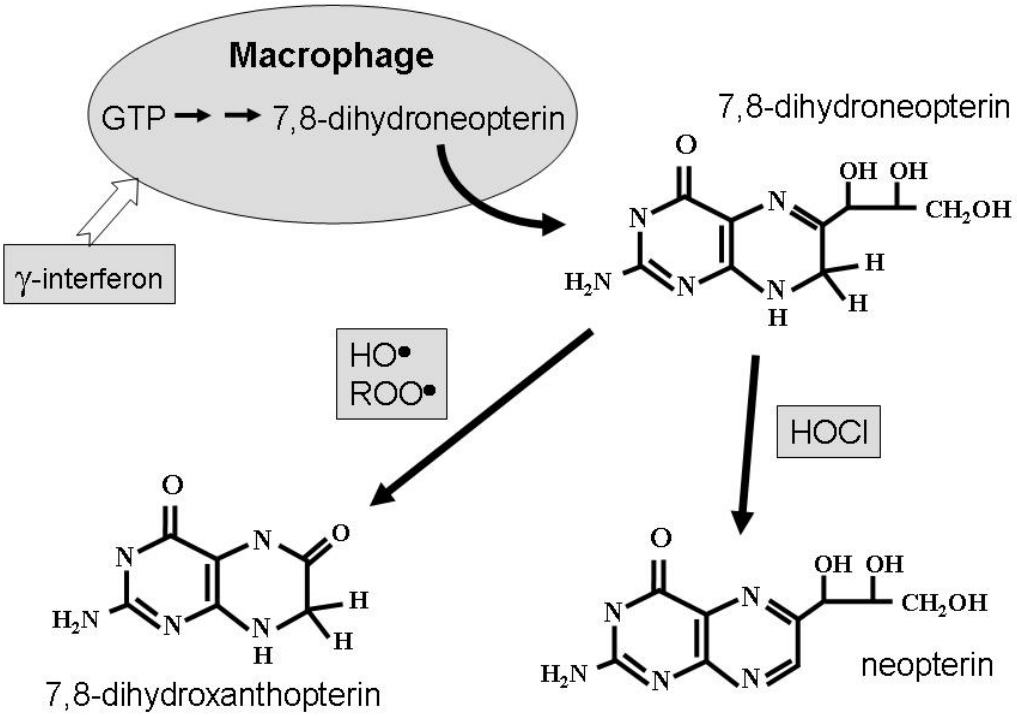


**Figure 5: Generation and oxidation of 7,8-dihydroneopterin.**

γ-Interferon stimulation of macrophages causes the enzymatic breakdown of intracellular GTP to 7,8-dihydroneopterin which can either be oxidised to the highly fluorescent neopterin by HOCl of to 7,8-dihydroxanthopterin by peroxyl and hydroxyl radicals.

disease, serum neopterin is elevated in atherosclerosis and correlates with the extent of the disease (Garcia-Moll et al. 2000, Tatzber et al. 1991, Weiss et al. 1994). The elevation is even more pronounced shortly after the onset of symptoms in patients with unstable angina and acute myocardial infarction (Auer et al. 2001, Schumacher et al. 1997).

Despite the clinical utility of neopterin as a marker of inflammation, the physiological role of both neopterin and 78NP is yet to be fully understood. Evidence for both pro- and antioxidative activities and cell signaling activities have been produced in numerous *in vitro* studies.

Antioxidative properties have been attributed to 78NP, although the first indicator of this action was demonstrated in another reduced neopterin derivative, tetrahydroneopterin, which showed high superoxide scavenging abilities in

xanthine/xanthine oxidase and phorbol myristate acetate stimulated macrophage systems (Kojima et al. 1992). 78NP was subsequently shown to have similar radical scavenging abilities through inhibition of chemiluminescence induced by hydrogen peroxide and chloroamine T (Weiss et al. 1993).

The ability of 78NP to protect LDL from oxidation was shown by an increased lag time in LDL oxidation mediated by $Cu^{2+}$ ions or aqueous peroxyl radicals (Gieseg et al. 1995). Low micromolar concentrations of 78NP have also been demonstrated to inhibit cell-mediated oxidation of LDL by THP-1 cells; a human derived monocyte-like cell line (Firth et al. 2007, Gieseg and Cato 2003).

78NP has also been shown to protect more complex target cells from free radical damage. Haemolysis of erythrocytes by hypochlorite and peroxyl radicals was prevented by 78NP (Gieseg et al. 2001a) and 78NP also provided a protective antioxidant effect to U937 cells (another monocyte-like cell line) against fenton generated reactive ROS and hypochlorite (Gieseg et al. 2001b) and TNF- α mediated apoptosis (Baier-Bitterlich et al. 1995). 78NP was also able to inhibit the toxic effects exerted by oxidised LDL on other cells by inhibiting thiol loss in U937 cells (Baird et al. 2005). This has lead to the hypothesis that 78NP is produced to protect the macrophages from oxidants during inflammation (Gieseg et al. 1995, Gieseg et al. 2000, Gieseg et al. 2007).

At much higher concentrations (>5mM) 78NP has been demonstrated to significantly enhance the TNF-α mediated apoptosis of U937 cells (Baier-Bitterlich et al. 1995) and neuronal NT2/HNT cells (Spottl et al. 2000). However, it is unlikely that 78NP would reach these concentrations *in vivo* as this is near its solubility limit. At 78NP concentrations of 1 mM or more apoptosis is induced in human peripheral blood T cells (Wirleitner et al. 2003). This programmed cell death could be mediated by the addition of the antioxidants catalase and superoxide dismutase and also by addition of a metal chelator. Apoptosis also occurs at these levels in Jurkat T cells (Baier-Bitterlich et al. 1996, Wirleitner et al. 1998, Wirlettner et al. 2001) and this has been shown to occur via the redox-sensitive Bcl-2 pathway (Enzinger et al. 2002a).

The mechanisms of this prooxidative effect of 78NP all involve direct generation of ROS due to the reducing power of 78NP (Oettl et al. 2004). This may be due to the reduction of metal ions within the cell media increasing ROS formation (Gieseg et al. 2007). This scenario only presents itself when the concentration of 78NP is extremely high and in protein-free media, as proteins are themselves excellent radical scavengers,

and thus may be of limited relevance within the atherosclerotic plaque (Gieseg et al. 2007).

Neopterin has also showed pro-apoptotic properties with low micromolar concentrations inducing apoptosis in rat alveolar epithelial cells (Schobersberger et al. 1996). Neopterin has been shown to exhibit enzyme inhibition action with the competitive inhibition of NADPH oxidase (Kojima et al. 1993) and has also been shown to stimulate iNOS gene expression and nitric oxide production in vascular smooth muscle cells and the synthesis of TNF-α (Hoffmann et al. 1998). It has also been shown to increase the singlet oxygen produced by MPO while decreasing other reactive species such as HOCl (Razumovitch et al. 2004). Additionally neopterin has been shown to inhibit ATP-induced calcium release in alveolar epithial cells (Hoffmann et al. 2002). As there is little evidence that neopterin is toxic to macrophages it has been hypothesised that neopterin augments the cytotoxic potential of activated macrophages (Hoffmann et al. 2003).

A hypothetical model for the actions of 78NP and neopterin within the atherosclerotic plaque has been proposed in which 78NP and neopterin exist in a pro/antioxidative balance (Gieseg et al. 2007). In the early stage of plaque formation 78NP would slow or inhibit oxLDL formation and prevent the oxLDL and hydroxyl radical damage of macrophages, which may contribute to the stabilization of foam cells. During the chronic inflammation of atherosclerosis lowered levels of 78NP would shift the redox balance to a pro-oxidative state leading to oxLDL formation and cell necrosis/apoptosis. Alternatively high levels of 78NP brought about by intense inflammation which would also result in increased HOCl formation would elevate neopterin levels which would switch off MPO activity and could trigger neutrophil apoptosis.

## Objectives of Study

The aim of this study is to examine oxidative damage and antioxidant content of atherosclerotic plaques and assess the relationship of these parameters to each other and to their localisation within the plaque.

A pilot study on atherosclerotic plaques performed previously in this lab has demonstrated the presence of neopterin in two atherosclerotic plaques and measured the concentrations of this, vitamin E and markers of protein and lipid oxidation across the length of the plaque. Prior to this measurement of neopterin has never been performed in atherosclerotic plaques. This study expands upon this data set allowing more powerful comparisons to be made.

All biochemical analysis studies of plaque over the past fifteen years have been carried out on whole plaque with no attempt to localise sites of differing biochemical conditions. This study allows for the identification of trends in oxidation levels and inflammatory markers in relation to spatial localisation within the plaque. Also many previous studies on plaque material remove matrix proteins by centrifugation and in doing so remove much of the analytes. Experimental methods employed in this study allow the analyses to be carried out on full plaque homogenate without the removal of important analytes.

With relation to measurement of neopterin in the plaque; weaknesses in the current method of neopterin and 78NP measurement in serum and tissue samples are addressed by the development of a new method for the precolumn treatment of the sample prior to HPLC analysis.

# Materials and Methods

## Materials

All chemicals and reagents used for this research were of analytical grade or better. Water was deionised and ultrafiltered using a NANOpure ultrapure water system (Barnstead/Thermolyne; Iowa, USA). All solutions were prepared in nanopure water. Salt-containing HPLC mobile phases were vacuum filtered through a 0.45 µM membrane and all HPLC mobile phases were degassed via sonication followed by online degassing.

### Chemicals and Reagents

| | |
|---|---|
| α-Tocopherol | Sigma Chemical Co.; St. Louis, MO, USA |
| 1,1,3,3-Tetramethoxypropane | Sigma Chemical Co. |
| 2,4-Dinitrophenyl hydrazine (DNPH) | Sigma Chemical Co. |
| 2-thiobarbituric acid (TBA) | Sigma Chemical Co. |
| 3,4-Dihydroxyphenylalanine (DOPA) | Sigma Chemical Co. |
| 7,8-Dihydroneopterin (78NP) | Schricks Laboratory; Switzerland |
| Acetone | Merck Ltd.; Darmstadt, Germany |
| Acetonitrile | Mallinckrodt Baker B.V.; Holland |
| Argon gas | BOC Gasses; NZ |
| Bicinchoninic acid (BCA) protein determination kit | Pierce; Illinois, USA |
| Bovine serum albumin (BSA) | Sigma Chemical Co. |
| Butylated hydroxytoluene (BHT) | Sigma Chemical Co. |
| Cholesterol reagent | Roche Diagnostics; USA |
| Di-ammonium hydrogen orthophosphate | BDH Laboratory Supplies; Poole, England |
| Diethyl ether | Merck Ltd. |
| Ethanol | Scharlau Chemie S.A.; Barcelona, Spain |
| Ethyl acetate | Merck Ltd. |
| Ethylenediaminetetraacetic acid (EDTA) | BDH Laboratory Supplies |
| Guanidine hydrochloride (GuHCl) | Sigma Chemical Co. |
| Hexane | Asia Pacific Specialty Chemicals |

| | |
|---|---|
| Hydrochloric acid (HCl) | Merck Ltd. |
| Iodine | BDH Laboratory Supplies |
| L-Ascorbic acid | BDH Laboratory Supplies |
| Mercaptoacetic acid | Sigma Chemical Co. |
| Methanol | Scharlau Chemie S.A. |
| Neopterin | Schricks Laboratory |
| Nitrogen gas | Southern Gas Services Ltd.; NZ |
| Orthophosphoric acid 85% | BDH Laboratory Supplies |
| Perchloric acid (PCA) | BDH Laboratory Supplies |
| Phenol | Sigma Chemical Co. |
| Potassium iodide | Merck Ltd. |
| Sodium dihydrogen orthophosphate | Scharlau Chemie S.A. |
| Trichloroacetic acid (TCA) | Sigma Chemical Co. |
| Trifluoroacetic acid (TFA) | Sigma Chemical Co. |

# Methods

## Sample Collection and Preparation

### Plasma Samples

Blood samples were obtained by venipuncture from ten randomly selected septicaemia patients and five healthy control volunteers. Septicaemia patients were undergoing amino glycoside therapy and blood samples were collected as part of their routine monitoring from the Department of Toxicology at Christchurch Hospital. Plasma was prepared by centrifugation and stored at -80°C until analysis for neopterin and total neopterin analysis.

### Plaque Samples

Plaques were removed from the carotid arteries of patients undergoing carotid endarectomies at the Department of Surgery at Christchurch Hospital. The plaque was placed on ice for transport to the Free Radical Biochemistry Laboratory where it was stored at -80°C. Written consent was obtained from the patients prior to surgery for analysis of their plaques and for the provision of clinical notes to the researcher. Plaques were assigned an identification number based on the patient's initials and date of surgery; this number is given at the beginning of the data for the individual plaques. For purposes of this publication plaques were arbitrarily assigned an alphabet letter for identification throughout the publication.

Prior to analysis the plaque was thawed and photographed. It was then dissected into sections of approximately 3-5 mm and the transverse view of the individual sections was photographed. The sections were numerically labeled in order starting proximal to the bifurcation. Each section was assigned to a zone (pre-bifurcation, bifurcation and post-bifurcation) depending on its localisation in the intact plaque. The plaque as a whole was classified based on qualitative visual observation by the researcher (heavily calcified, thrombosed or neither).

Weights of the sections were recorded prior to homogenisation with a tissumizer in 5 mL water and 50 µL each of 20 mg/mL BHT (in methanol) and 100 mg/mL EDTA (pH 7.4). The total volume of homogenate was recorded before samples were aliquoted

for analysis of carbonyls, cholesterol, dityrosine, DOPA, neopterin, protein, TBARS and vitamin E.

## HPLC Analyses

High Performance Liquid Chromatography (HPLC) measurements were performed using a Shimadzu Sil-10A HPLC system with temperature controlled autosampler and RF-10Axls fluorescence detector (Shimadzu Corporation; Japan). Peak areas were quantified using the Shimadzu CLASS-VP software package.

### Neopterin and Total Neopterin

Neopterin was detected in both plaque and plasma samples by its native fluorescence at 438 nm, excitation 353 nm. Total neopterin (neopterin plus 78NP) was measured by first oxidising the 78NP to neopterin (Werner et al. 1987). Two differing sample preparation methods were employed for neopterin measurement; the original method involving acid precipitation of proteins using TCA (Werner et al. 1987), and a new method which employed acetonitrile for protein precipitation developed as part of this research program. Due to the limited amount of plaque homogenate available, plaques were only measured by either the TCA method or the acetonitrile method.

TCA Method

100 µL of sample was combined with 10 µL of 6 µM ascorbate and 10 µL of 50% trichloroacetic acid (TCA) followed by vortex then centrifugation ( $4^{\circ}$C and 10 300g for 15 min ); 100 µL of the acid supernatant was then placed in an autosampler vial for HPLC analysis.

Acetonitrile Method

100 µL of sample was combined with 100 µL of 100% acetonitrile, vortexed and centrifuged ($4^{0}$C and 10 300g for 10 min). 100 µL of the supernatant was then transferred to an autosampler vial for HPLC analysis.

Total Neopterin

For total neopterin analysis an oxidation step was included to convert 78NP to neopterin for detection following the protein precipitation and centrifugation steps. After following the methods of protein precipitation outlined above 10 µL of acidic iodide

solution (5.4% $I_2$ / 10.8% KI in 1 M HCl) was added to supernatant and incubated for 20 minutes at room temperature in the dark. 10 µL of 6 µM ascorbate was added to oxidise the iodine before centrifugation of the samples ($4^0$C and 10 300g for 5 min). With the TCA treatment method for total neopterin analysis the ascorbate was not added before protein precipitation as it is when measuring neopterin alone but added subsequent to iodide oxidation of 78NP as described above.

HPLC Analysis

10 µL of sample was injected onto a Phenomenex Develosil reverse phase ODS-MG-5 4.2 x 250 mm column and developed isocratically with a mobile phase of 5% methanol in 20 mM ammonium phosphate pH 6.0 pumped at 1 mL/minute (Gieseg et al. 2001a). The eluting neopterin was detected by fluorescence at excitation 353 nm and emission 438 nm. The column temperature was maintained at 35°C. The concentration and identity of the eluted neopterin was confirmed by comparison to a commercial standard of 1µM neopterin (Schricks Laboratory; Switzerland) and quantified by peak area.

**TBARS**

The TBARS assay is an indirect measure of lipid peroxidation. The reaction of 2-thiobarbituric acid (TBA) with the lipid hydroperoxide breakdown product, malondialdehyde (MDA), produces a pink adduct which can detected fluorometrically (Draper et al. 1993).

100 µL of plaque homogenate was combined with 50 µL of 150 mM phosphoric acid and a further 10 µL of 20 mg/mL BHT (in methanol). The samples were then stored at -80°C while awaiting analysis.

The 42mM TBA reagent is prepared fresh on the day of analysis by dissolving TBA in water while heating to approximately 50°C, after which it is allowed to cool before use. After thawing, samples underwent a 30 minute incubation at 95°C in the presence of 50 µL of 42 mM TBA reagent. The samples were then cooled on ice and centrifuged (4°C, 10 300g, 10 minutes).

20 µL of supernatant was injected onto a Phenosphere reverse phase C-18 4.6 x 150 mm 5 µm column and developed isocratically with a mobile phase of 45% methanol in 50 mM sodium dihydrogen phosphate pH 6.8 pumped at 1 mL/minute. The eluting

TBARS were detected by fluorescence at excitation 525 nm and emission 550 nm. The column temperature was maintained at 30°C.

The concentration and identity of the eluted TBARS was confirmed by comparison to the peak areas of 0µM and 1µM MDA standards reacted with TBA as outlined above. The MDA standard was prepared from 6mM 1,1,3,3-tetramethoxypropane in 2:3 ethanol:water, subsequently diluted in water to the appropriate concentrations which is thermolytically hydrolysed during the 95°C incubation. Phosphoric acid and BHT were included in the standards as per the experimental samples.

**Vitamin E**

α-Tocopherol (vitamin E) was determined by reverse phase HPLC with fluorescence detection of hexane extracts of plaque homogenate (Esterbauer et al. 1987, Gieseg et al. 1995).

100 µL of plaque homogenate was Gieseg et al. 1995a combined with a further 400 µL of water, 10 µL of 100 mg/mL EDTA (pH 7.4) and 25 µL of 20 mg/mL BHT in methanol. Protein was precipitated with 500 µL of ethanol and the vitamin E was extracted into 2 mL of hexane (60 second vortex). Samples were stored at -80°C while awaiting analysis. Upon thawing samples were vortexed for a further 60 seconds before centrifugation (4°C, 1 600g, 5 minutes) to maximise phase separation. 1400 µL of the upper hexane phase was dried under nitrogen gas and the resulting residue was redissolved in 50 µL of methanol for HPLC analysis.

20 µL of sample was injected onto an Econosphere reverse phase C-18 4 x 125 mm 5 µm column and developed isocratically with a mobile phase of 100% methanol at 1 mL/minute. The eluting Vitamin E was detected by fluorescence at excitation 292 nm and emission 335 nm. The column temperature was maintained at 35°C. The concentration and identity of the eluted vitamin E was confirmed by comparison to a 3µM Vitamin E standard (Sigma Chemical Co.; USA) and quantified by peak area.

**DOPA and Dityrosine**

DOPA and dityrosine are oxidation products of the amino acid tyrosine and thus are a measure of protein oxidation. Protein-bound DOPA and dityrosine are measured by

their native fluorescence via reverse phase gradient HPLC following the acid hydrolysis of the protein into free amino acids (Gieseg et al. 1993).

In glass Durham tubes 100 µL of plaque homogenate was mixed with 900 µL of glacial acetone and placed on ice for 10 minutes before centrifugation. The protein pellet was washed with 500 µL of glacial diethyl ether and centrifuged as above. The diethyl ether was drained off with any residual removed under vacuum leaving the pellet ready for acid hydrolysis. This procedure removes all free lipid, salt and carbohydrates which can interfere with the analysis.

Samples were placed into Pico-Tag reaction vials (Millipore; USA) containing 1 mL of 6 M HCl with 1% ($^{w}/_{v}$) phenol and 50 µL mercaptoacetic acid. Vials were flushed with argon gas for 10 minutes and then evacuated using a vacuum pump. Samples were incubated for 16 hours at 110°C. The resulting hydrolysis residue was dried in a speed vac and then redissolved in 200 µL of 0.1% TFA for HPLC analysis.

10 µL of sample was injected onto a Phenomenex reverse phase C-18 250 x 4.6 mm 5 µm column with a mobile phase of 0.1% TFA pH 2.5 pumped at 1 mL/minute. The sample was separated by a gradient which started at 0% Acetonitrile rising to 5% over 10 minutes. The acetonitrile was increased to 10% over the next 4 minutes and further increased to 50% over the next 4 minutes. The acetonitrile content was then decreased to 1% over seven minutes before finally decreasing the acetonitrile to 0% over the next 7 minutes.

The eluted DOPA and dityrosine were detected by fluorescence at excitation 280 nm and, initially, emission 320 nm for DOPA detection. After 13 minutes the emission wavelength was changed to 410 nm for dityrosine detection. The column temperature was maintained at 35°C. The concentrations and identities of the eluted DOPA and dityrosine were confirmed by being compared to a commercial standard (Sigma Chemical Co.; USA) and a previously prepared laboratory standard (Amado et al. 1984) respectively, and quantified by peak area.

## UV Visible Spectrometry

### Carbonyls

Carbonyls are a general measure of protein oxidation. The reaction of carbonyls with 2,4-dinitrophenyl hydrazine (DNPH) forms a carbonyl-DNPH derivative with maximal absorbance at 360 nm (Quinlan et al. 1994).

200 uL of plaque homogenate was combined with either 1 mL of 10 mM DNPH in 2 M HCl or 1 mL of 2 M HCl only to serve as a blank. All samples were incubated with gentle shaking for 90 minutes at 37°C and were then cooled on ice in the presence of 1 mL of 28% TCA. Samples were centrifuged (4°C, 4 100g, 10 minutes) and the resulting pellet was washed twice with 5 mL of 1:1 ethanol:ethyl acetate. The dried pellet was resuspended in 1 mL of 6 M guanidine hydrochloride in 2 M HCl and incubated for one hour at room temperature in the dark to allow colour to develop.

Samples were centrifuged (4°C, 4 100g, 5 minutes) and the absorbancies of the supernatant were recorded at 360 nm. Blank values were subtracted from the corresponding DNPH treatment value and the carbonyl concentration was calculated using an extinction co-efficient of 21,000 $cm^{-1}.M^{-1}$ (Quinlan et al. 1994).

### Total Cholesterol

Total cholesterol content was determined by incubation of 10 µL of plaque homogenate with 1 mL of enzymatic “Chol. MPR 2” reagent (Roche Chemicals) for 30 minutes. Absorbancies were recorded at 500 nm against a cholesterol reagent blank and cholesterol content calculated as per the manufacturers’ instructions.

### Protein

Protein concentration was determined used a bicinchonic acid (BCA) protein determination kit: “working reagent” was freshly prepared by combining Reagent A (sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0.1 M sodium hydroxide) and Reagent B (4% hydrated copper sulphate) in a 50:1 ratio.

Plaque homogenate was diluted with water to appropriate levels and 50 µL was mixed with 1 mL of “working reagent”. Samples were incubated for 30 minutes at 60°C with gentle shaking, after which the samples were placed on ice to halt the reaction.

Absorbancies were recorded at 562 nm and the protein concentration was determined from a standard curve of commercial BSA supplied with the kit.

## Statistics

All analyses were carried out on triplicate samples (unless otherwise stated). Concentrations given are the mean of triplicates with standard error shown as a way one error bar.

Statistics were performed with the software packages Statistica (version 7.1; StatSoft, Inc., USA) and Prism (version 4.0; Graphpad Software, USA).

Between sample comparisons were performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test to designate significant variations between means. Significance levels are indicated within the text.

Factorial ANOVA was also performed on plaque data; prior to which the data sets were assessed for homogeneity of variances and normality. Data not conforming to these assumptions was transformed accordingly before analysis. Analyses containing significant variation was presented as the least square of the mean (LS mean) by the relevant factor with error bars denoting the 95% confidence intervals.

Pearson correlation analyses were performed on plaque data and significant relationships were presented in tabular form.

## Ethics

Ethics approval for the analysis of plaque and access to clinical patient data was given by the Upper South Ethic Committee; ethics approval number 01/04/036. All blood samples were surplus to clinical requirements and were anonymised before release for neopterin analysis as part of the diagnostic laboratory ethics approval for assay validation or establishing a reference range. This study was been approved of by the Upper South A Regional Ethics Committee; ethics approval number URA/07/16/EXP.

# Results

## Neopterin Analysis Development

HPLC analysis of plasma or tissue requires the removal of proteins. Original methods used ion exchange solid phase extraction to collect and concentrate the neopterin (Fukushima and Nixon 1980, Werner et al. 1987), but with changes to solid phase manufacturing these methods appear to have become unreliable and technically demanding. The more common methods now employed remove sample proteins by acid precipitation prior to HPLC analysis. However, under acidic conditions, 7,8-dihydroneopterin (78NP) is oxidised to neopterin with varying yield (Figure 6) (Werner et al. 1987). This oxidation can be partially prevented by the addition of ascorbate prior to acid precipitation but is not completely effective (Figure 6). Acetonitrile was assessed as an alternate, non-acidic reagent for protein precipitation.

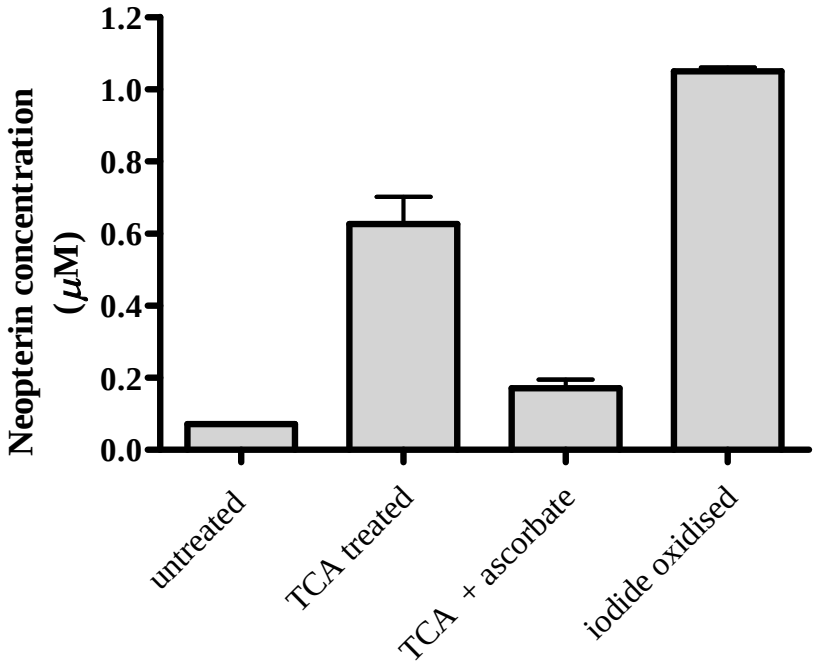


**Figure 6: Oxidation of 78NP to neopterin by TCA.**

Standard solutions of 1µM 78NP were analysed for neopterin content. The samples were left untreated, were fully oxidised to neopterin using an acidic iodide solution (iodide oxidised) or 100µl of standard was treated with 10µl of 50% TCA (TCA treated) or 10µl of 50% TCA and 10µl of 6µM ascorbate (TCA + ascorbate) prior to HPLC analysis. Values shown represent the mean + SE of triplicates.

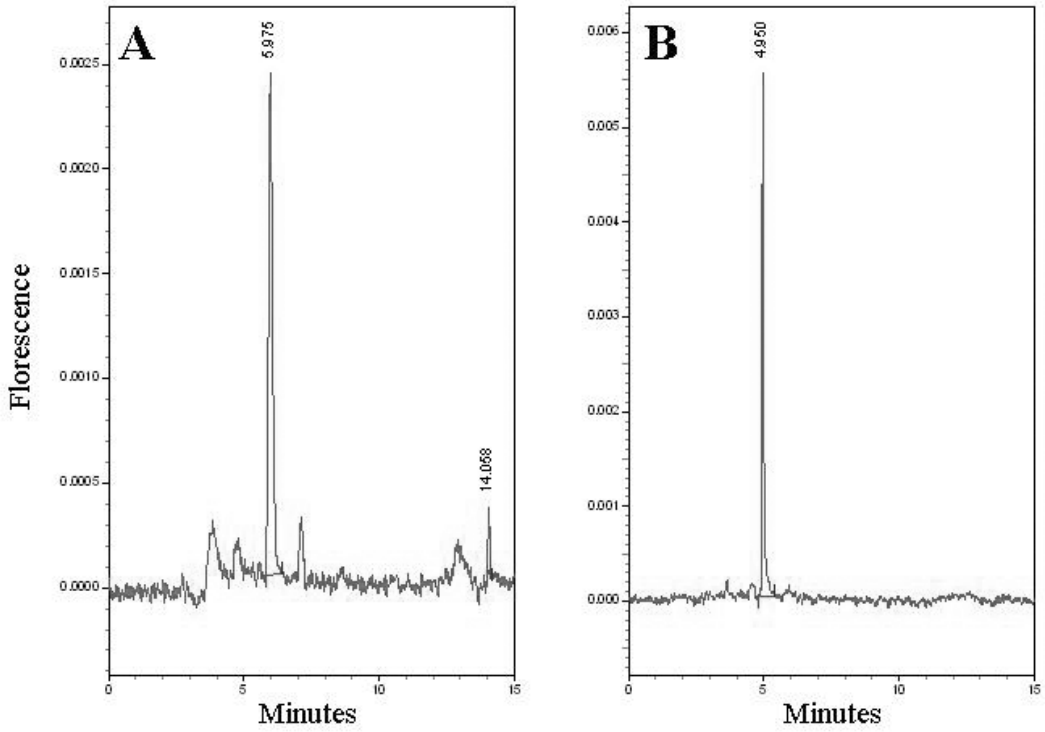


**Figure 7: HPLC chromatograms of plasma neopterin.**

HPLC chromatograms of neopterin from plasma samples treated prior to injection with either A) TCA or B) acetonitrile. Samples were taken from a selected septicaemia patient. Note the scale in B is 0.0 to 0.006 compared to 0.0 to 0.0025 in A.

The use of acetonitrile to remove sample proteins markedly improved the resolution and signal to noise ratio seen during HPLC compared to TCA treatment (Figure 7). TCA treated samples also showed a number of additional contaminant peaks during chromatography. With the acetonitrile treated samples, neopterin was consistently observed to elute one minute sooner than the neopterin from TCA treated samples. This is due to the increase in organic solvent in the samples from the addition of 50% acetonitrile. The neopterin peak identity was confirmed by spiking plasma samples with authentic neopterin.

In the acetonitrile treated sample's chromatogram (Figure 7B), the neopterin peak area is smaller than that seen in the chromatogram of the TCA treated sample (Figure 7A). This is due to the dilution of sample which occurs with the acetonitrile treatment. When dilution factors are taken into account the apparent concentration of neopterin in the plasma from the TCA treated sample is 31.4 nM and in the acetonitrile treated sample

55.3 nM. This apparent increase in neopterin levels with acetonitrile treatment was generally observed with all plasma and tissue samples examined. This increase in neopterin with acetonitrile treatment was not observed using protein free buffers suggesting the effect was due to the actual precipitation of the protein.

The linearity of the acetonitrile treatment and analysis was confirmed by measuring different concentrations of neopterin between 0.05 and 2 µM. The calibration curve ($y = 1.019x – 0.015$) was linear over this range with a correlation coefficient of $r^2 = 1.00$. Within-run precision, evaluated by ten consecutive injects from the same plasma sample, and between-run precision, evaluated by injects on six separate days from the same plasma pool, showed repeatability of the assay is good (within-run CV% < 2.9, between-run CV% < 3.8).

Analysis of ten septicemia patients and five apparently healthy controls showed the acetonitrile treatment consistently returned higher levels of neopterin and total neopterin than the acid based treatment (Figure 8 & Figure 9). The acetonitrile precipitation treatment gave on average a 20 nM increase in neopterin levels compared to that obtained using TCA for both the healthy controls and septicemia patients (Figure 8A & Figure 9A). However, with total neopterin analysis (78NP + neopterin), where the 78NP is oxidised to neopterin, the increase in neopterin due to the acetonitrile treatment, compared to TCA, was less consistent. With healthy controls acetonitrile treatment gave on average a 50% increase in total neopterin compared to TCA, but this increase ranged from 4.3 nM to 12.9 nM (Figure 9B).

With septicemia patients the measured total neopterin level also increased with acetonitrile treatment (Figure 9B) but by approximately 100% compared to the TCA treated samples. The size of this increase ranged from 15 nM to 120 nM.

TCA treatment shows a picture of healthy subjects having the majority of the pterin as 78NP (75%) (Figure 10). In the septicemia patients, this ratio is reversed where the majority of the pterin is present as neopterin (83%).

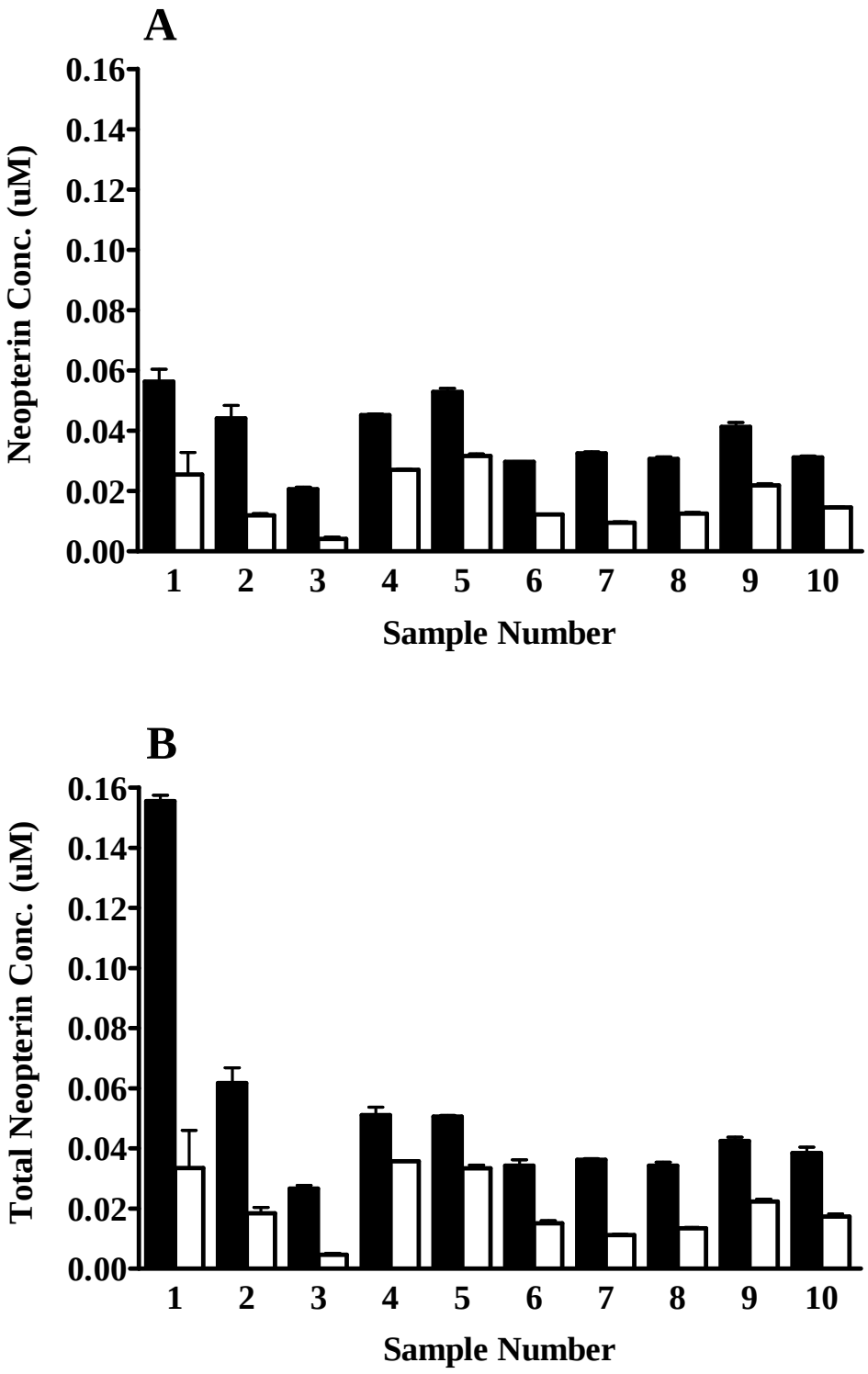


**Figure 8: Effect of sample preparation on plasma neopterins from septicaemia patients.**

Plasma neopterin (A) and total neopterin (B) concentrations for ten septicaemia patients. Samples were prepared for analysis using either acetonitrile (■) or TCA (□) for protein precipitation before HPLC analysis as described in methods. Values graphed are the mean + SE of three replicates for each sample.

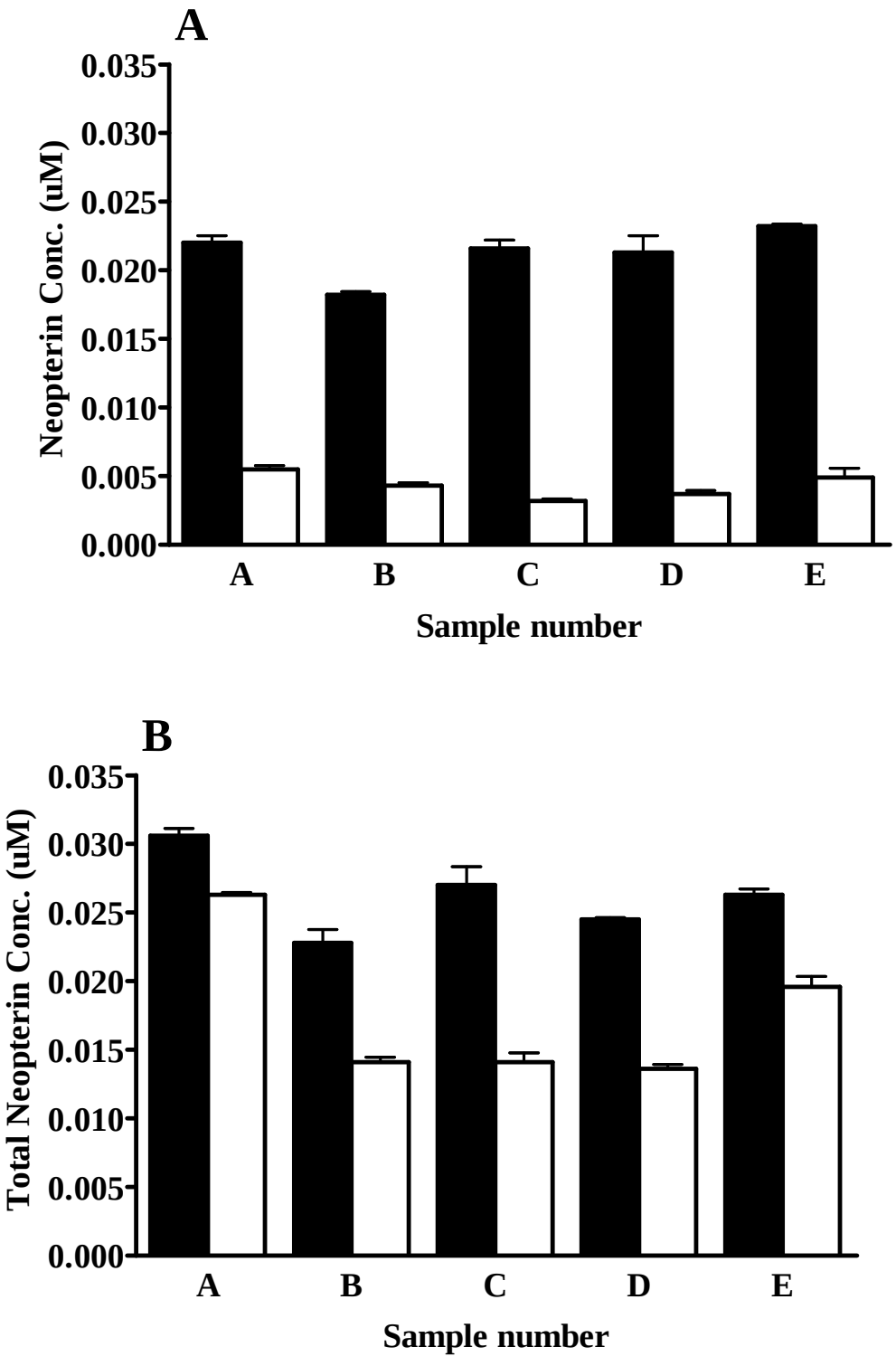


**Figure 9: Effect of sample preparation on plasma pterins from healthy volunteers.**

Plasma neopterin (A) and total neopterin (B) concentrations for five apparently healthy volunteers. Samples were prepared for analysis using either acetonitrile (■) or TCA (□) for protein precipitation before HPLC analysis as described in methods. Values graphed are the mean + SE of three replicates for each sample.

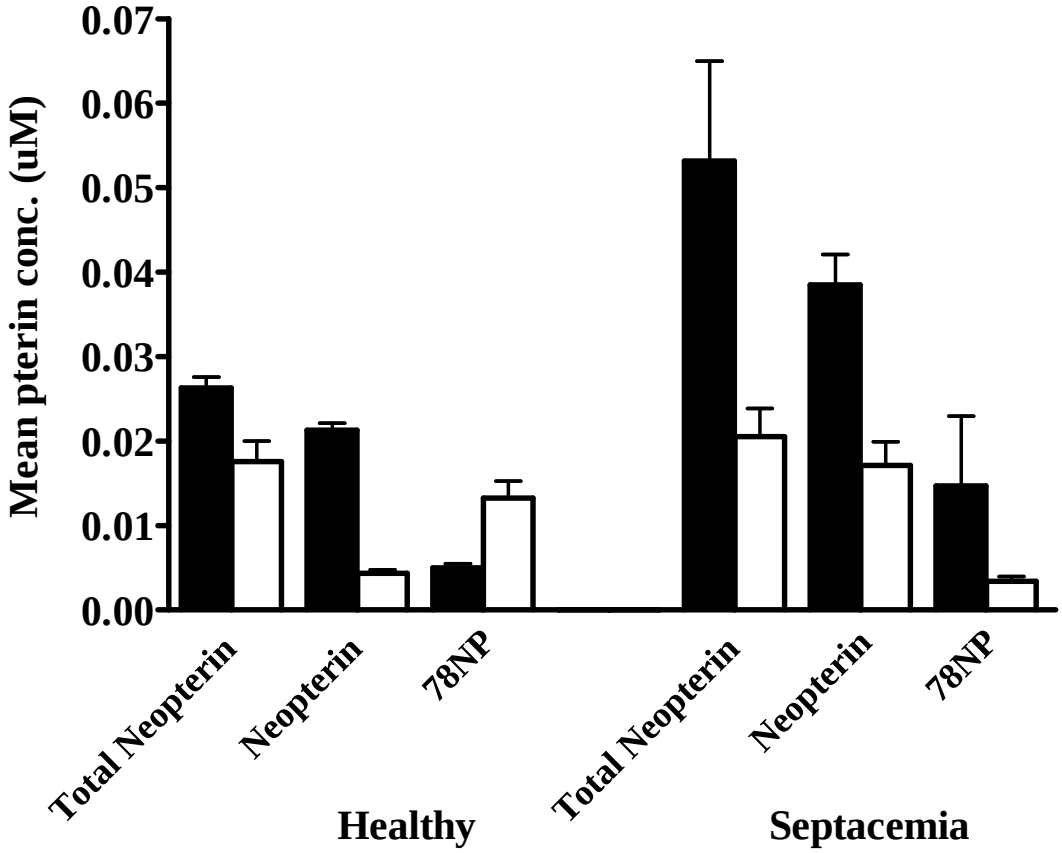


**Figure 10: Comparison of average plasma pterin concentrations from different sample preparations.**

Mean plasma total neopterin, neopterin and 78NP concentrations for the two experimental groups (septicaemia and healthy controls). The 78NP concentration for each sample was calculated by subtracting the total neopterin concentration from the plasma neopterin concentration. The figure shows the mean of the acetonitrile (■) and TCA (□) treated samples from Figure 8 and Figure 9.

# Oxidation and Inflammation in Atherosclerotic Plaques

## Patient Information

A total of eight patients participated in this study. Participants had an average age of 73 years and were either ex- or non-smokers. None of the patients were diabetic and all patients were taking aspirin for their condition. Plaques removed all showed a high degree of stenosis, ranging from 65 to 90 percent, as this is a prerequisite of the surgery. Patients suffered from various symptoms including hypertension, transient ischemic attacks (TIA), stroke, and amaurosis fugax (temporary loss of vision). Patients suffering from hypertension were receiving medications for this symptom (one or a combination of the following: lorsatan, frusemide, inhibace, cilazapril, bendofluazide and candesartan). Other medications prescribed varied from patient to patient and were classified as either statins or beta blockers. Statins prescribed to one or more patients were atorvastatin and simvastatin. The prescribed beta blocker was metaprolol. Medications not related to their condition are not included in the patient summary. Data for the individual patients is summarised in Table 1. Classification of plaques was based on qualitative observation by

**Table 1: Characteristics of carotid endarterectomy patients and plaques.**

| Patient/ Plaque | Sex | Age (years) | Plaque classification | Stenosis % | Symptoms: | | | | Medication: | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Hyper-tension | TIA | Stroke | Amaurosis fugax | Statin | Beta blocker |
| A | F | 76 | neither | 80 | | ✓ | | | ✓ | |
| B | F | 71 | thrombosed | 90 | | ✓ | ✓ | | ✓ | ✓ |
| C | M | 71 | calcified | 70 | ✓ | ✓ | | | ✓ | |
| D | F | 64 | thrombosed | 75 | ✓ | | | ✓ | ✓ | ✓ |
| E | F | 72 | calcified | 90 | ✓ | ✓ | ✓ | | | |
| F | M | 67 | thrombosed | 80 | ✓ | | | ✓ | | ✓ |
| G | M | 80 | neither | 80 | | ✓ | | ✓ | | |
| H | M | 83 | calcified | 65 | ✓ | | ✓ | ✓ | ✓ | |

the researcher and was placed into one of three categories: thrombosed, heavily calcified, or neither. This classification along with the percent of stenosis for each plaque is also given in Table 1.

## Compiled Plaque Data

Six plaques (A-F) were analysed and data for two further plaques (G – DB011203 & H – AC100903) were taken from previous analyses performed by Carole Firth (Firth 2006)

Markers of protein oxidation which includes DOPA, dityrosine and carbonyls have been expressed per gram of protein, as this is of greater consequence as a limiting factor compared with plaque mass as a whole and removes the variable of protein concentration which may mask or exaggerate the actual concentrations in the different sections. This idea was also applied to TBARS as a measure of lipid oxidation and the lipid soluble vitamin E, thus they were presented as per gram of cholesterol. Raw data presented per gram of plaque is attached in appendix I.

**Table 2: Data averages for individual plaques**

| **Plaque** | **Total Mass** (g) | **Protein** (mg/g plaque) | **Cholesterol** (µmole/g plaque) | **Carbonyls** (µmole/g protein) | **Dityrosine** (nmole/g protein) | **DOPA** (nmole/g protein) | **Neopterin*** (nmole/g plaque) | **TBARS** (nmole/ mole cholesterol) | **Vitamin E** (µmole/ mole cholesterol) |
|---|---|---|---|---|---|---|---|---|---|
| A | 1.50 | 70.3 | 75.8 | 4.79 | 9.35 | 245.18 | 0.087* | 115.08 | 512.2 |
| B | 0.76 | 92.0 | 80.9 | 5.00 | 149.26 | 340.54 | 0.211* | 86.41 | 1030.8 |
| C | 1.31 | 64.3 | 58.0 | 4.43 | 14.14 | 79.08 | 1.588 | 77.34 | 1594.5 |
| D | 0.82 | 92.1 | 47.4 | 4.82 | 101.76 | 179.90 | 1.795 | 178.08 | 3073.4 |
| E | 0.51 | 94.8 | 25.8 | 3.02 | 0.00 | 84.59 | 1.262 | 607.54 | 1640.8 |
| F | 0.38 | 140.3 | 59.3 | 2.76 | 66.85 | 72.05 | 0.921 | 255.53 | 2737.0 |
| G | 0.90 | 77.6 | 37.2 | 2.23 | 5.84 | 170.77 | 0.207* | 128.1 | 582.6 |
| H | 2.86 | 67.1 | 92.4 | 5.62 | 68.37 | 182.04 | 0.158* | 48.57 | 458.6 |

* Neopterin in plaques A, B, G and H were measured using the TCA protein precipitation method; plaques C, D, E and F were measured using the acetonitrile protein precipitation method.

## Plaque A – IH120704

Approximately 37 mm of plaque material was removed from the right internal carotid artery of patient A (Figure 11). It was dissected into eight segments of 3-6 mm each. The plaque showed neither a high degree of calcification nor evidence of thrombosis so was classified as neither. For purposes of between plaque comparisons, section 1 was assigned as pre-bifurcation, 2-4 as bifurcation and sections 5-8 as post-bifurcation.

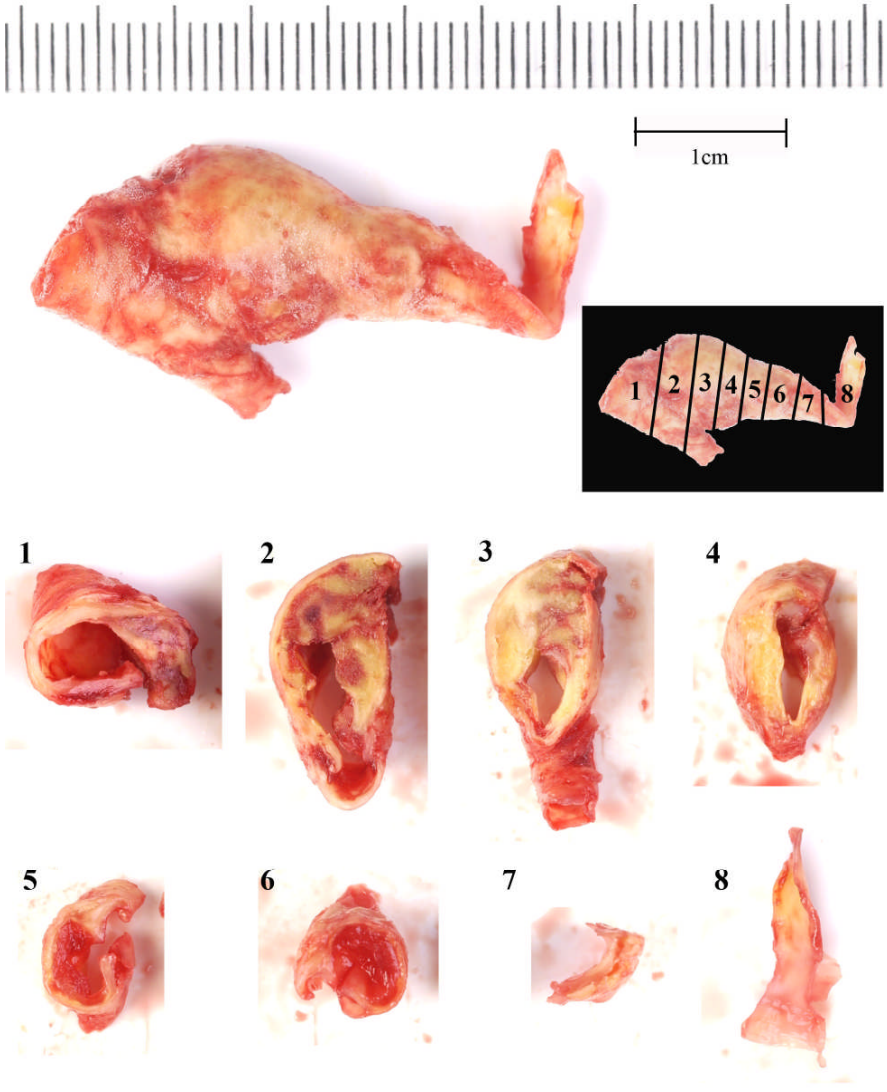


**Figure 11: Plaque A - Cross sections of the atheromatous material removed from the carotid artery of patient A.**

The plaque was separated and removed from the right internal carotid artery of patient A. It was dissected into eight segments labelled sections 1-8 as shown. Section 1 was assigned as pre-bifurcation, sections 2-4 as bifurcation and sections 5-8 as post-bifurcation.

**Protein and Cholesterol**

Cholesterol content of plaque A shows higher levels of cholesterol ($p<0.05$) to be present in the bifurcation and just down stream of the bifurcation in sections 2 to 5 (Figure 12B).

Due to a shortage of homogenate material protein for plaque A was only able to be measured in duplicate; consequently any differences in the protein concentration can not be described as statistically significant. However observation of the data (Figure 12A) does show a trend of higher protein levels in earlier sections of the plaque which tends to decrease as we precede downstream of the bifurcation.

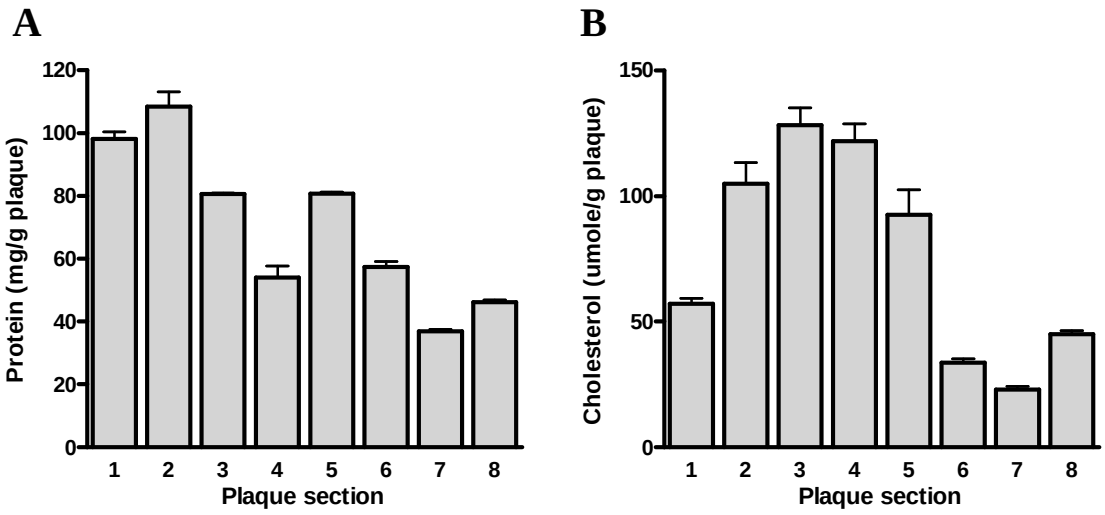


**Figure 12: Protein and cholesterol concentrations in eight sections of plaque A.**

Protein content (A) was analysed in individual sections using a BCA protein determination kit. Cholesterol content (B) was determined in each section by spectrophotometer. Values shown represent the mean + SE of triplicates for cholesterol and duplicates for protein.

**Neopterin**

Neopterin levels in plaque A were measured using the TCA protein precipitation method. The average neopterin concentration across all sections of the plaque was 0.087 nmoles/g of plaque. The individual sections show a significant peak ($p < 0.05$) in the first, pre-bifurcation section and a significant trough in the final section, with the remaining sections' neopterin concentration falling in between these levels (Figure 13).

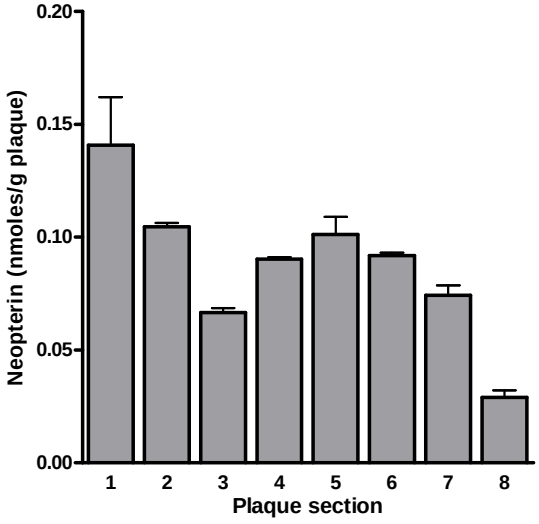


**Figure 13: Neopterin concentration in eight sections of plaque A.**

Individual sections of plaque A were treated with TCA to precipitate proteins prior to HPLC analysis for neopterin. Values shown represent the mean + SE of triplicates.

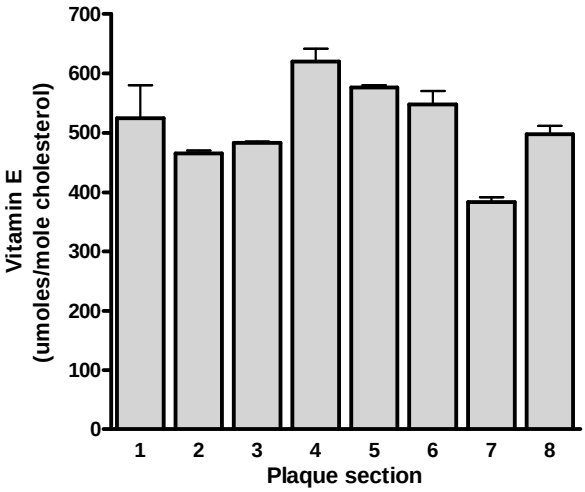


**Figure 14: Vitamin E concentration in eight sections of plaque A.**

Individual sections of plaque A were analysed using HPLC for their vitamin E content. Values shown represent the mean + SE of triplicates.

**Vitamin E**

A significant peak in vitamin E is present in section 4 of plaque A and section 7 represents a significant trough (Figure 14). The plaque did not, however, show any apparent trend in the localisation of vitamin E.

**Markers of Oxidation**

DOPA maintained a consistent concentration of approximately 200 nmoles/g of protein in the first six sections of plaque A, after which it increased significantly ($p<0.05$), to almost twice that value in the tail sections (Figure 15A).

In contrast to DOPA, the later sections, 7 and 8, contained no detectable dityrosine; the majority of dityrosine being present in the pre- and bifurcation sections, with a peak of 21.9 ±1.1 nmoles/g of protein in section 3 (Figure 15B).

Carbonyls did not reveal any obvious trends and, apart from section 4, there was no significant difference between any of the sections ($p<0.05$); section 4 representing a peak of 8.72 ± 0.17 µmoles/g of protein for plaque A (Figure 15C).

TBARS showed a slight trend of higher concentrations in the later, post-bifurcation sections with sections 6 and 7 representing a significant peak ($p<0.05$) (Figure 15D).

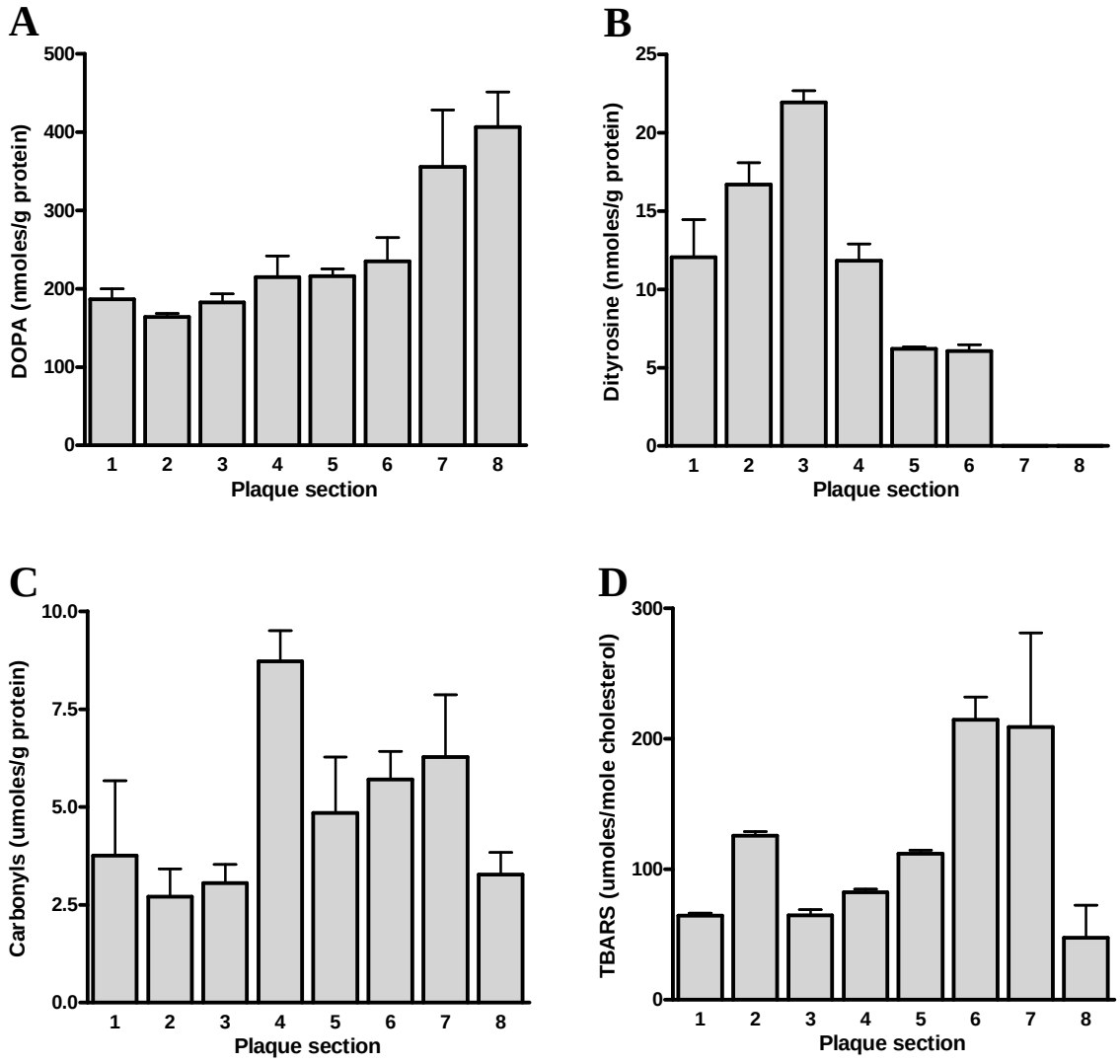


**Figure 15: Markers of oxidation in eight sections of plaque A.**

Individual sections of plaque A were analysed for DOPA (A), dityrosine (B), carbonyl (C) and TBARS (D) content. Values shown represent the mean + SE of triplicates.

## Plaque B – HW290304

Approximately 25 mm of plaque material was removed from the left carotid artery of patient B (Figure 16). It was dissected into seven segments ranging from 0.08-0.13 g in weight. The plaque showed evidence of a major thrombic event, with section 7 consisting entirely of a blood clot, and thus was placed in the thrombosis classification. For purposes of between plaque comparisons, sections 1 and 2 were assigned as pre-bifurcation, 3-5 as bifurcation and section 6 as post-bifurcation. As section 7 did not contain any actual plaque material, it was not included in the between plaque analyses; however, the data was included in the individual plaque data.

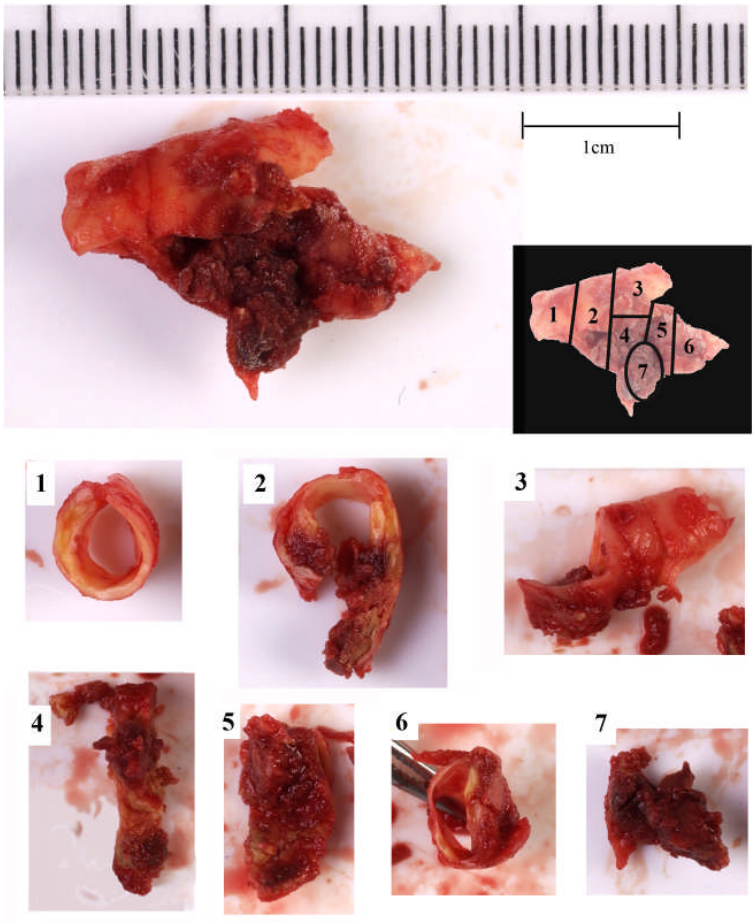


**Figure 16: Plaque B - Cross sections of the atheromatous material removed from the carotid artery of patient B.**

The plaque was separated and removed from the left carotid artery of patient B. It was dissected into seven segments labelled sections 1-7 as shown. Sections 1-2 were assigned as pre-bifurcation, sections 3-5 as bifurcation and section 6 as post-bifurcation. Section 7 was a blood clot and for purposes of between-plaque analysis was not included in the data set however the data is shown for the individual plaque.

**Protein and Cholesterol**

Protein concentration per gram of plaque was similar across the majority of the plaque sections apart from a significant ($p<0.01$) spike in section 6, at the tail end of the plaque B (Figure 17A).

Cholesterol content in the leading edge of plaque B is significantly ($p<0.01$) lower then subsequent sections, with a value of just 25.5 ± 5.4 µmoles/g of plaque, it is less then half that of any other section (Figure 17B). Cholesterol levels in the blood clot (section 7) are higher; and overall the blood clot shows a higher cholesterol to protein ratio then the plaque tissue.

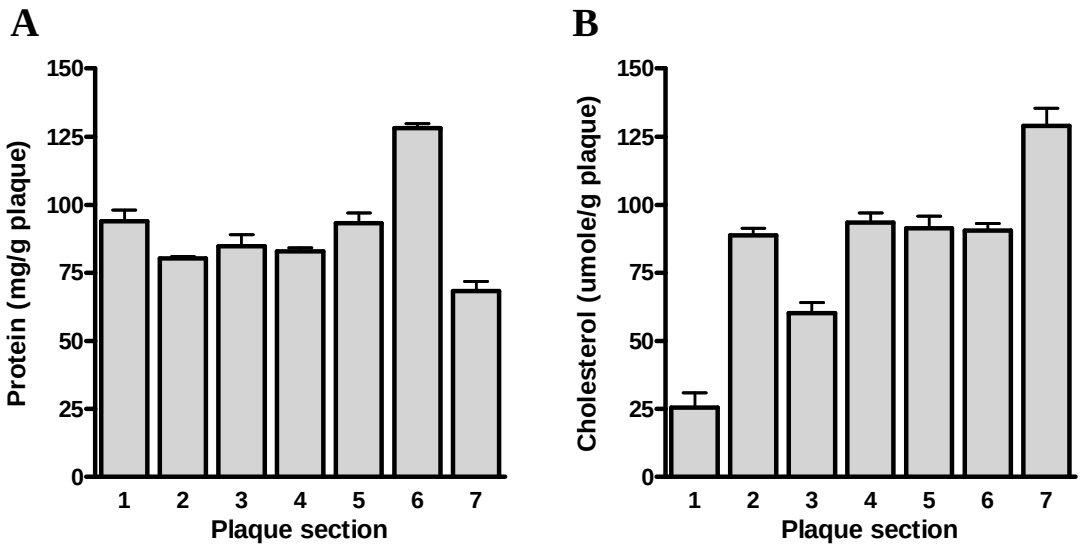


**Figure 17: Protein and cholesterol concentrations in seven sections of plaque B.**
Protein content (A) was analysed in individual sections using a BCA protein determination kit. Cholesterol content (B) was determined in each section by spectrophotometer. Values shown represent the mean + SE of triplicates.

**Neopterin**

Neopterin levels in plaque B were measured using the TCA protein precipitation method. The concentration of neopterin in the pre-bifurcation zone and the distal end of the plaque are considerably higher ($p<0.001$) then the areas of, and directly after, the bifurcation (Figure 18). The average neopterin concentration of 0.211 nmoles/g of plaque is more then doubled in the peak concentration of section 2.

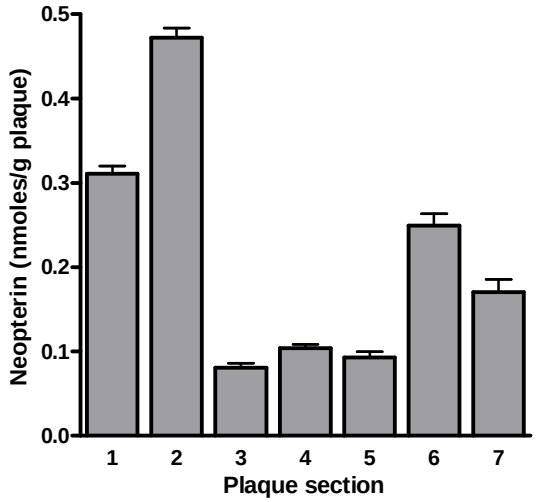


**Figure 18: Neopterin concentration in seven sections of plaque B.**

Individual sections of plaque B were treated with TCA to precipitate proteins prior to HPLC analysis for neopterin. Values shown represent the mean + SE of triplicates.

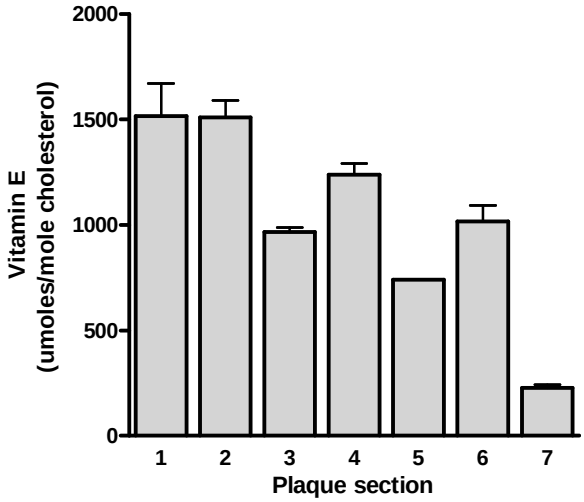


**Figure 19: Vitamin E concentration for seven sections of plaque B.**

Individual sections of plaque B were analysed for their vitamin E content by HPLC. Values shown represent the mean + SE of triplicates.

**Vitamin E**

A trend of decreasing vitamin E concentration is apparent as we move from the proximal end of plaque B downstream (Figure 19). The pre-bifurcation sections (1 and 2) are significantly higher ($p<0.05$) then section 3, 5 and 6. The blood clot at section seven has a considerably ($p<0.05$) lower concentration of vitamin E in relation to all the sections containing plaque tissue.

**Markers of oxidation**

DOPA concentration is greatest at the proximal end at a concentration of 606 ± 120 nmoles/g of protein, the majority of the later sections being significantly lower then this value ($p<0.05$), with a trend of lower levels in the post-bifurcation section (Figure 20A).

Dityrosine, on the other hand, is at its lowest concentration for plaque B in section 1 (Figure 20B). Instead reaching a significant ($p<0.05$) peak at the bifurcation in section 4, before dropping again in the post bifurcation sections.

The variation in the data for carbonyls means that no significant trends can be postulated from this data (Figure 20C). TBARS had a similar problem with large variation in replicates, however, when data was pooled into zones (bifurcation, pre-bifurcation, etc) the bifurcation sections were shown to be significantly higher ($p<0.01$) then the pre- and post-bifurcation sections (Figure 20D).

Section 7, the blood clot, shows medium to high levels of all the oxidative markers compared to other sections (Figure 20). Most notably, the value of 625 ± 24 nmoles of dityrosine/g of protein (Figure 20B), for exceeds the values obtained in plaque tissue for this, or any other, plaque.

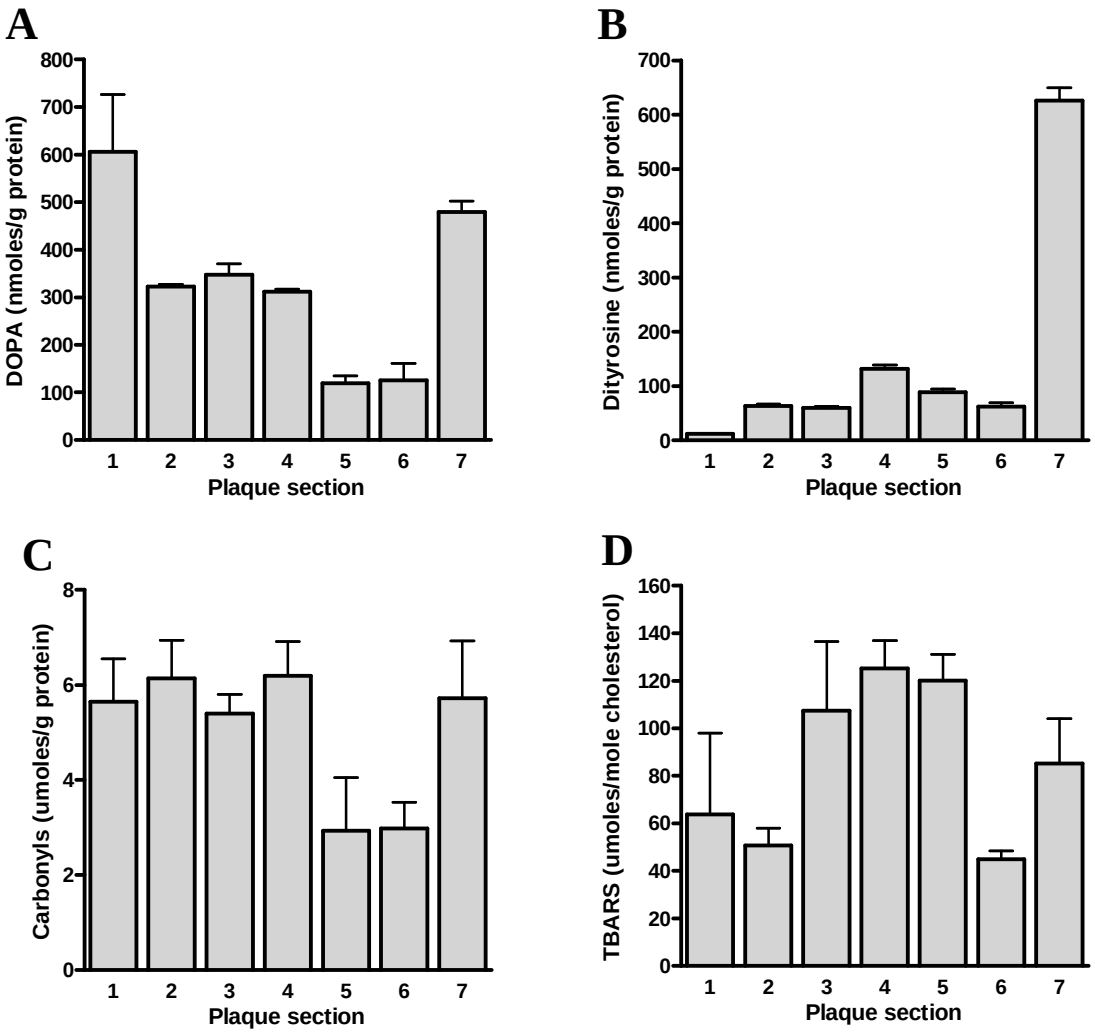


**Figure 20: Markers of oxidation in seven sections of plaque B.**

Individual sections of plaque B were analysed for their content of: DOPA (A), dityrosine (B), carbonyl (C) and TBARS (D). Values shown represent the mean + SE of triplicates.

## Plaque C – WC210604

Approximately 31 mm of plaque material was removed from the left carotid artery of patient C (Figure 21). It was dissected into seven segments ranging from 0.11-0.27 g in weight. The plaque was classified as highly calcified, as it was very solid and crunchy during dissection and homogenisation, particularly sections 2 and 4. For purposes of between plaque comparisons, sections 1 and 2 were assigned as pre-bifurcation, 3-5 as bifurcation and sections 6 and 7 as post-bifurcation.

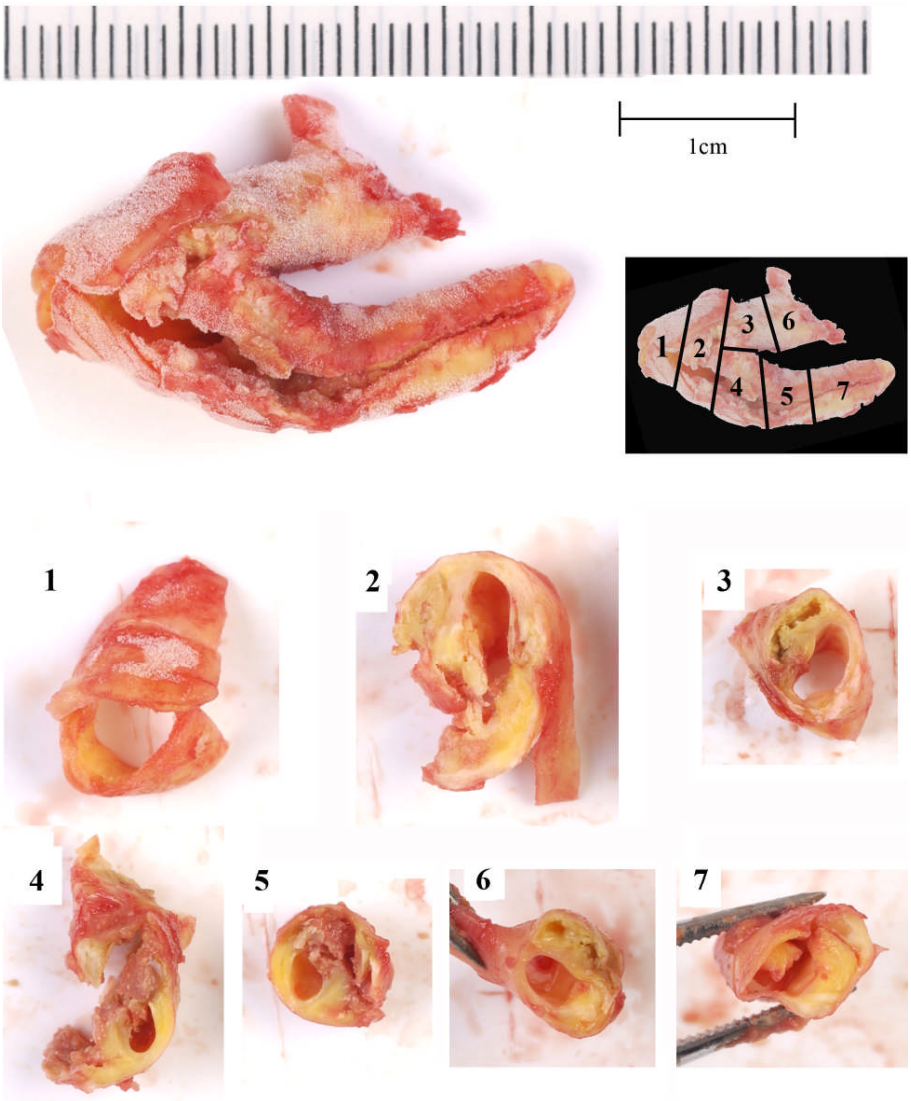


**Figure 21: Plaque C - Cross sections of the atheromatous material removed from the carotid artery of patient C.**

The plaque was separated and removed from the left carotid artery of patient C. It was dissected into seven segments labelled sections 1-7 as shown. Sections 1-2 were assigned as pre-bifurcation, sections 3-5 as bifurcation and sections 6-7 as post-bifurcation.

**Protein and cholesterol**

Protein concentration per gram of plaque is reasonably consistent throughout the entire plaque, with section 3 representing a significant ($p<0.05$) peak at 78.5 ± 5.7 nmoles/g of plaque (Figure 22A).

Cholesterol content in the leading edge of plaque C is significantly lower ($p<0.001$) then the later of the plaque (Figure 22B). Sections 3 and 5, localised in the bifurcation, represent significant peaks ($p<0.001$) in cholesterol concentration.

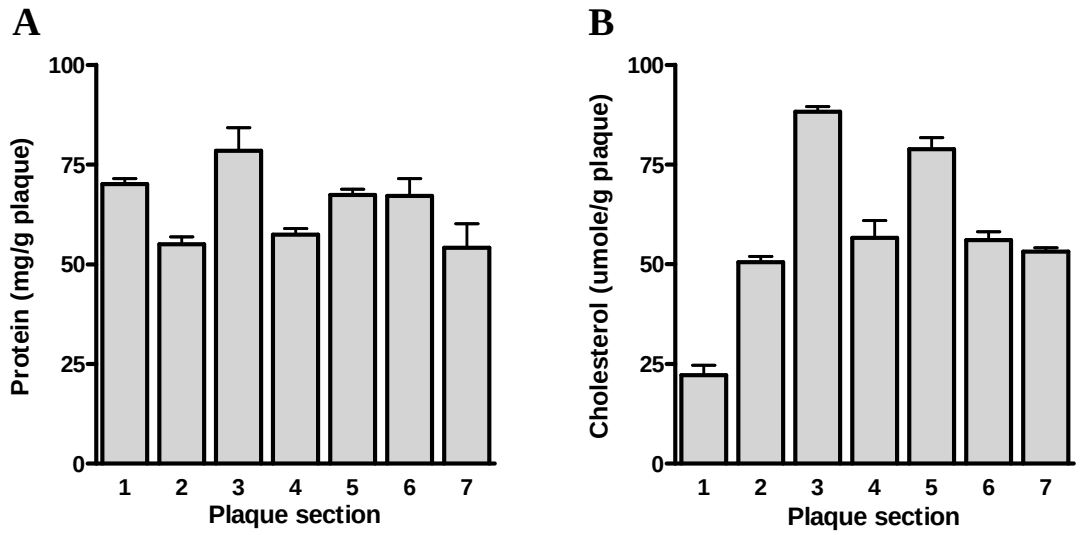


**Figure 22: Protein and cholesterol concentrations in seven sections of plaque C.**

Protein content (A) was analysed in individual sections using a BCA protein determination kit. Cholesterol content (B) was determined in each section by spectrophotometer. Values shown represent the mean + SE of triplicates.

**Neopterin**

Neopterin levels in plaque C were measured using the ACN protein precipitation method. Neopterin within plaque C did not demonstrate any real pattern in regard to localisation of the sections, with significantly high and low neopterin concentrations in each of the pre-, post- and bifurcation zones ($p<0.001$) (Figure 23).

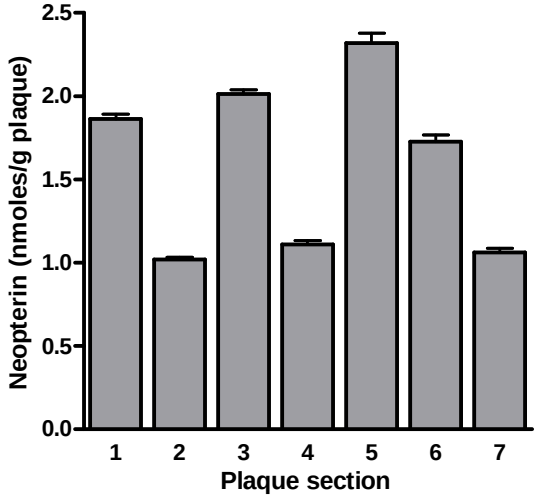


**Figure 23: Neopterin concentration in seven sections of plaque C.**

Individual sections of plaque C were treated with ACN to precipitate proteins prior to HPLC analysis for neopterin. Values shown represent the mean + SE of triplicates.

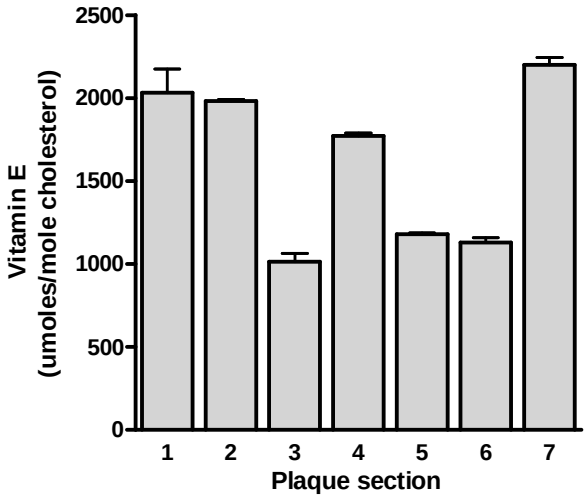


**Figure 24: Vitamin E concentration in seven sections of plaque C.**

Individual sections of plaque C were analysed using HPLC for their vitamin E content. Values shown represent the mean + SE of triplicates.

**Vitamin E**

Vitamin E showed significantly low ($p<0.001$) concentrations per gram of cholesterol in sections 3 and 6 representing the secondary branch of the bifurcation along with section 5 (Figure 24). Higher values are found pre-bifurcation in sections 1 and 2, and in the distal section of the primary branch (section 7).

**Markers of oxidation**

The peak in DOPA of 181 ± 27 nmoles/g of protein in section 2 of the pre-bifurcation zone represents the only significant ($p<0.05$) variation in DOPA concentration for plaque C (Figure 25A).

Dityrosine shows a trend of low concentrations in the proximal and distal ends of the plaque C with significant ($p<0.05$) increases in dityrosine content as we move inwards towards the bifurcation of the plaque, peaking at 32 ± 2 nmoles/g of protein in section 4 (Figure 25B).

As with DOPA, the only significant ($p<0.05$) variation in carbonyls concentration is in the pre-bifurcation zone of the plaque, with a peak in section 1 of 7.4 ± 0.7 µmoles/g of protein (Figure 25C).

Plaque C shows very high levels ($p<0.01$) of TBARS in both section 4 of the bifurcation and in the distal end of the primary branch (section 7) (Figure 25D). In contrast the sections representing the secondary branch (sections 3 and 6) show significantly ($p<0.05$) low concentrations, as do the sections upstream of the bifurcation.

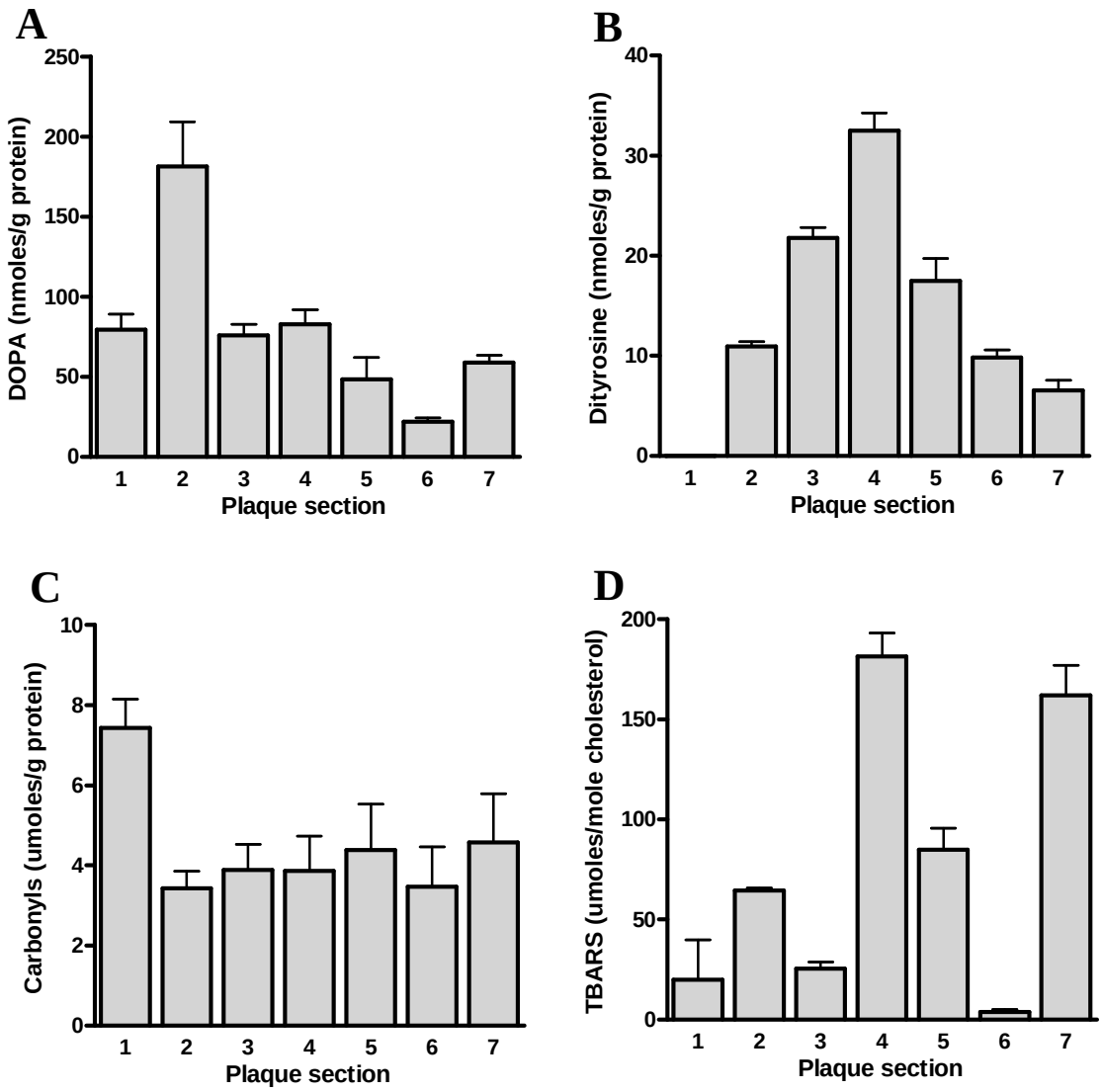


**Figure 25: Markers of oxidation in seven sections of plaque C.**

Individual sections of plaque C were analysed for their content of: DOPA (A), dityrosine (B), carbonyl (C) and TBARS (D). Values shown represent the mean + SE of triplicates.

## Plaque D – MS200405

Approximately 27 mm of plaque material was removed from the common carotid artery of patient D (Figure 26). It was dissected into six segments ranging from 0.06-0.27g in weight. The plaque was classified as thrombosed as blood clotting was evident throughout the plaque, especially in section 3, 4 and 5. For purposes of between plaque comparisons, sections 1 and 2 were assigned as pre-bifurcation, 3 and 4 as bifurcation and sections 5 and 6 as post-bifurcation.

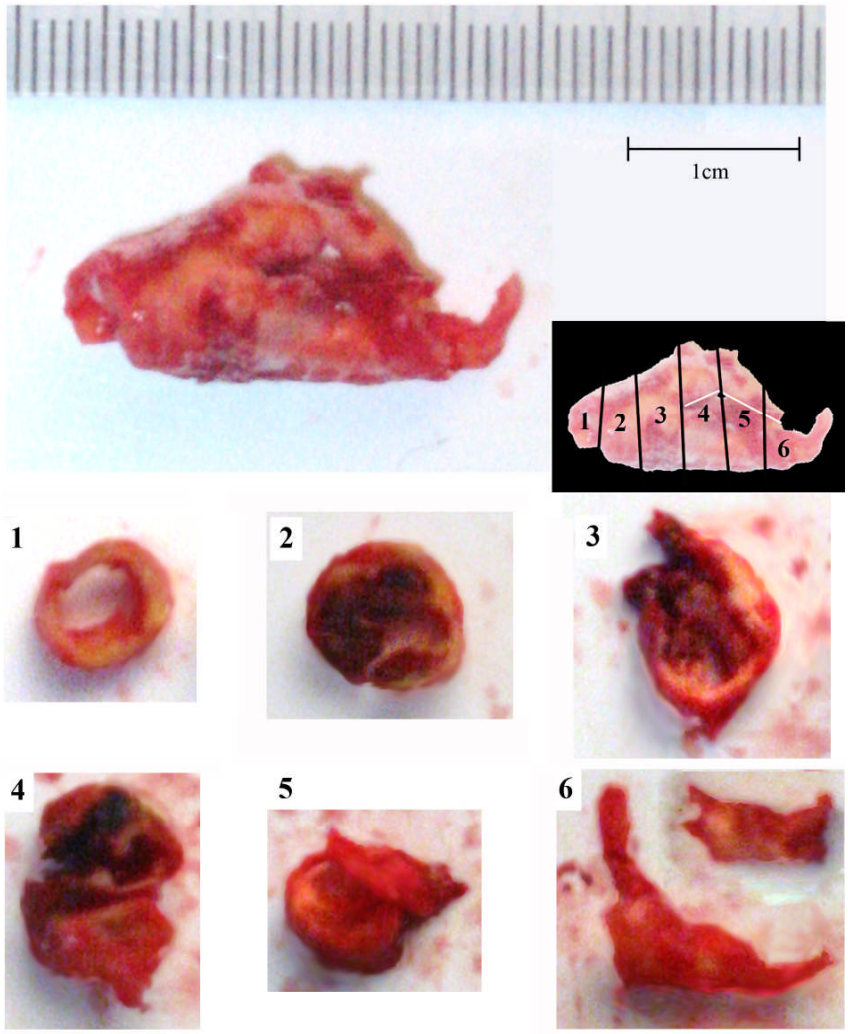


**Figure 26: Plaque D - Cross sections of the atheromatous material removed from the carotid artery of patient D.**

The plaque was separated and removed from the common carotid artery (side not stated) of patient D. It was dissected into six segments labelled sections 1-6 as shown. Sections 1-2 were assigned as pre-bifurcation, sections 3-4 as bifurcation and sections 5-6 as post-bifurcation. Note the camera normally used for photography was not available at time of dissection, thus the lower quality image.

**Protein and cholesterol**

Protein content in plaque D is significantly lower ($p<0.05$) within sections 2 and 4, and peaks within section 3 (Figure 27A). The protein concentration fluctuates within the pre- and bifurcation zones, thus no trend in protein concentration is apparent.

The post-bifurcation region of plaque D shows significantly ($p<0.05$) higher cholesterol content in plaque D then that seen upstream of and within bifurcation sections.

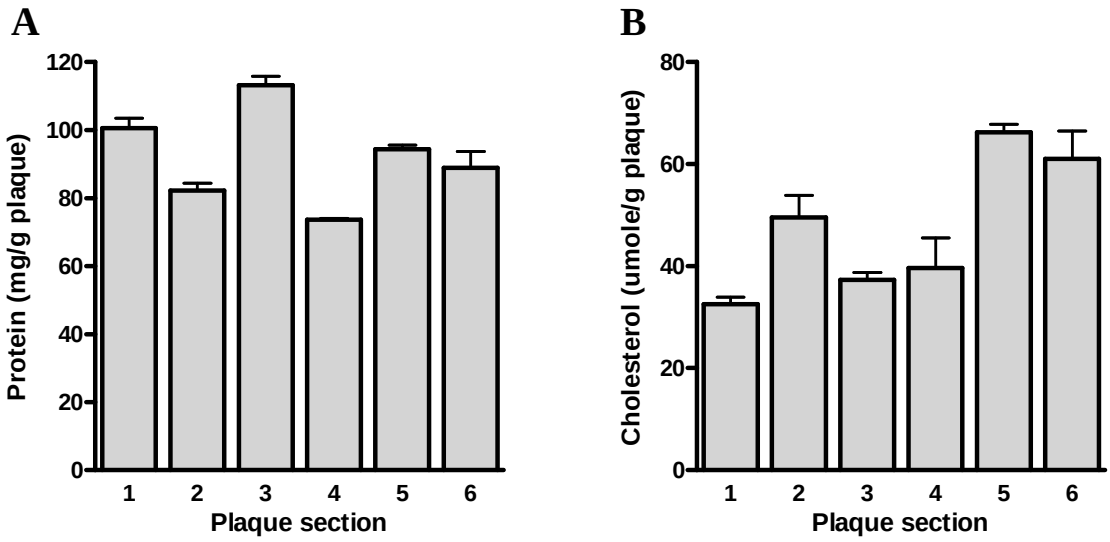


**Figure 27: Protein and cholesterol concentrations in six sections of plaque D.**

Protein content (A) was analysed in individual sections using a BCA protein determination kit. Cholesterol content (B) was determined in each section by spectrophotometer. Values shown represent the mean + SE of triplicates.

**Neopterin**

Neopterin levels in plaque D were measured using the ACN protein precipitation method. The proximal end of the plaque showed a significantly greater ($p<0.001$) level of neopterin then that present in the rest of the plaque with a concentration of 2.98 ± 0.09 nmoles/g of plaque, almost double that seen in other sections (Figure 28). Section 4 represents a significant trough ($p<0.001$) at the bifurcation zone.

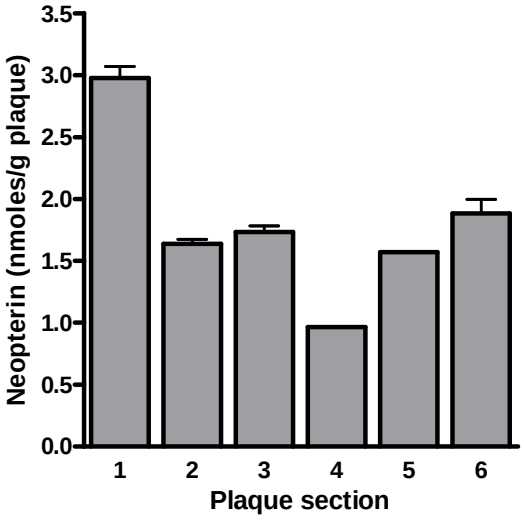


**Figure 28: Neopterin concentration in six sections of plaque D.**

Individual sections of plaque D were treated with ACN to precipitate proteins prior to HPLC analysis for neopterin. Values shown represent the mean + SE of triplicates.

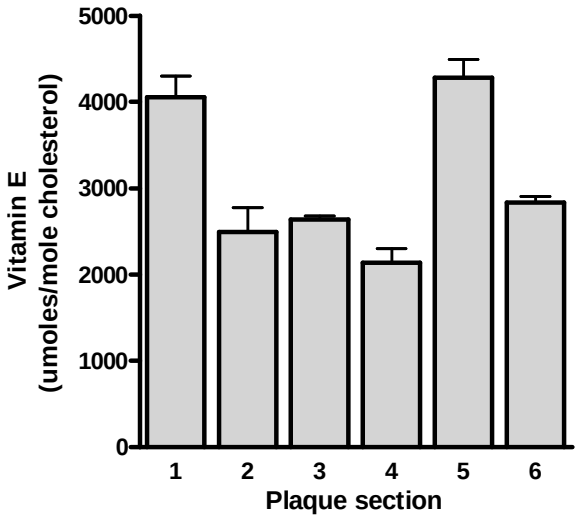


**Figure 29: Vitamin E for six sections of plaque D.**

Individual sections of plaque D were analysed using HPLC for their vitamin E content. Values shown represent the mean + SE of triplicates.

**Vitamin E**

Sections 1 and 5 represent significant peaks ($p<0.01$) in vitamin E localised within the pre-bifurcation and post-bifurcation zones respectively (Figure 29). The remaining sections of plaque D have similar vitamin E concentrations of approximately 2.5 mmoles/mole of cholesterol.

**Markers of oxidation**

Large variation in some of the replicates for DOPA prevent much of the differences between sections from being significant, however the two sections with the lowest DOPA content, 1 and 5, are significantly different from section 2 ($p<0.05$), and a general trend of lower levels of DOPA are found in the post-bifurcation zone (Figure 30A).

There were no detectable levels of dityrosine in section 1 of plaque D, however this was followed by a large increase in concentration in section 2, similar to that

observed in the DOPA samples (Figure 30B). Dityrosine peaks significantly ($p<0.05$) in section 4 at 213 ± 6 nmoles/g of protein, and then drops again drastically section 6.

The average carbonyl content for plaque D is 4.8 µmoles/g of protein and there is no significant variation between the any of the sections due to the large variation between replicates (Figure 30C).

TBARS shows a general trend of significantly higher levels ($p<0.05$) at the proximal end of plaque D decreasing to almost nothing at the distal end (Figure 30D).

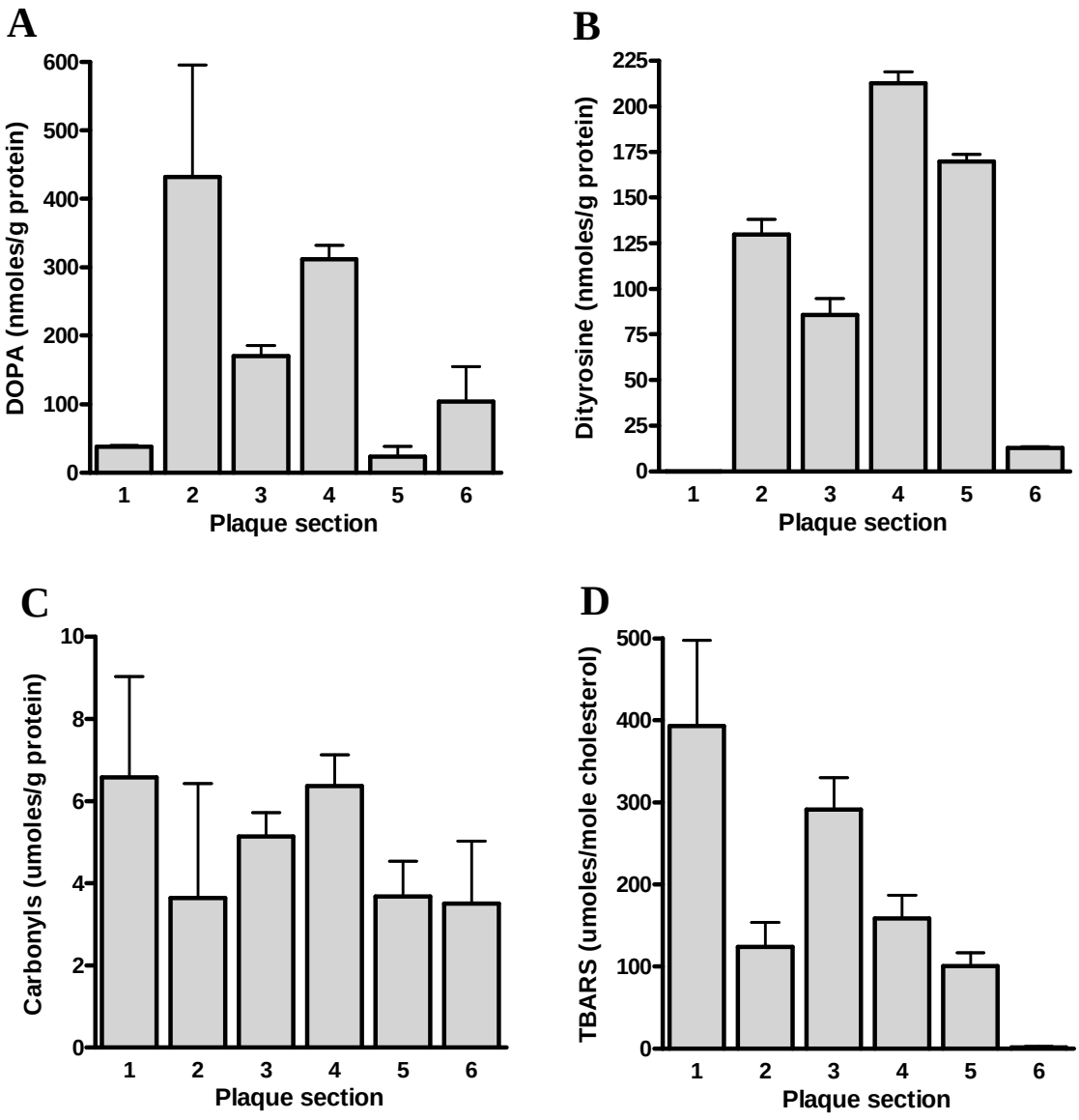


**Figure 30: Markers of oxidation in six sections of plaque D.**

Individual sections of plaque D were analysed for their content of: DOPA (A), dityrosine (B), carbonyl (C) and TBARS (D). Values shown represent the mean + SE of triplicates.

## Plaque E – ES020407

Approximately 18 mm of plaque material was removed from the left common carotid artery of patient E (Figure 31). It was dissected into six segments ranging from 0.03-0.18 g in weight. The plaque was classified as heavily calcified as it was very solid and crunchy during dissection and homogenisation, especially in sections 2, 3, and 5. For purposes of between plaque comparisons, sections 1 and 2 were assigned as pre-bifurcation, 3-5 as bifurcation and section 6 as post-bifurcation.

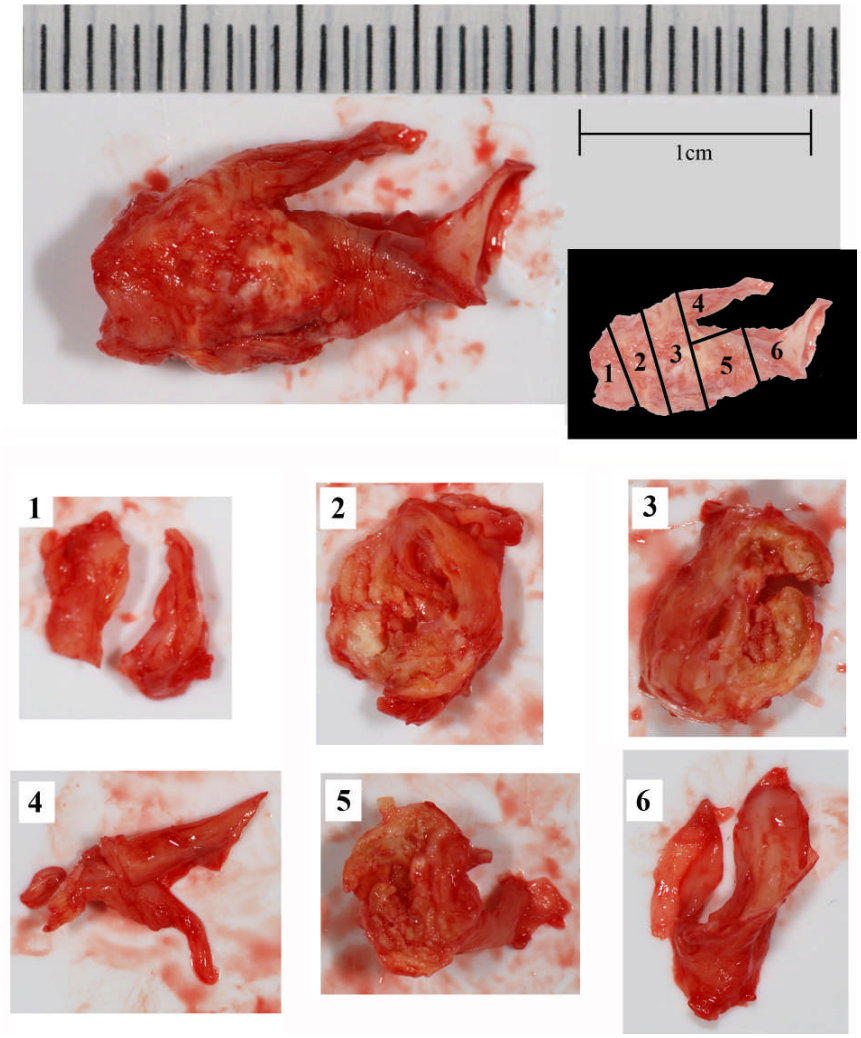


**Figure 31: Plaque E - Cross sections of the atheromatous material removed from the carotid artery of patient E.**

The plaque was separated and removed from the left common carotid artery of patient E. It was dissected into six segments labelled sections 1-6 as shown. Sections 1-2 were assigned as pre-bifurcation, section 3-5 as bifurcation and section 6 as post-bifurcation.

**Protein and cholesterol**

The marginal sections (1, 4 and 6) of plaque E show considerably higher concentrations ($p<0.05$) of protein per gram of plaque then those making up the body of the plaque (Figure 32A). This will be due, in part, to the large content of calcified gruel observed in sections 2, 3 and 5 contributing to the mass of these sections.

Cholesterol in section 2 is significantly higher ($p<0.01$) then in all other sections of plaque E (Figure 32B). The remaining sections are all within a similar range with section 4 representing a peak and 3 a trough within these sections ($p<0.05$). No trend related to localisation of the sections or observed calcium content is readily apparent for cholesterol in this plaque.

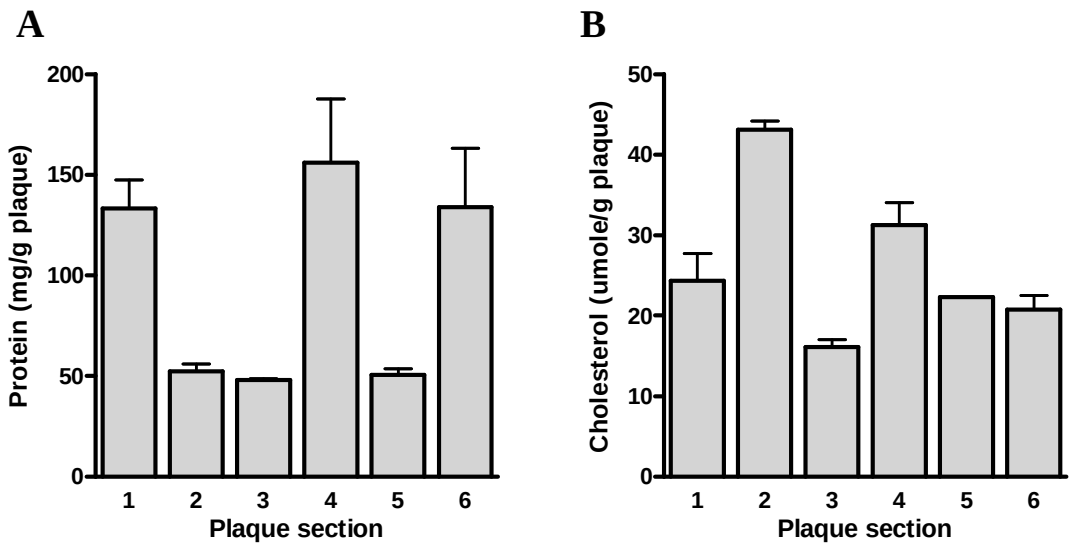


**Figure 32: Protein and cholesterol concentrations in six sections of plaque E.**

Protein content (A) was analysed in individual sections using a BCA protein determination kit. Cholesterol content (B) was determined in each section by spectrophotometer. Values shown represent the mean + SE of triplicates.

**Neopterin**

Neopterin in plaque E was measured using the acetonitrile protein precipitation method. A pattern of neopterin content (Figure 33) almost identical to that observed for protein localization is evident. Again the sections at the margins of the plaque contain significantly greater concentrations ($p<0.001$) of neopterin then those in the core With a higher level also measured in the distal sction at the start of the external carotid artery.

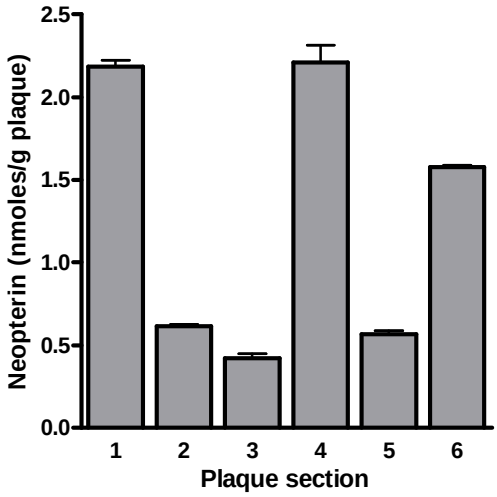


**Figure 33: Neopterin concentration in six sections of plaque E.**

Individual sections of plaque E were treated with ACN to precipitate proteins prior to HPLC analysis for neopterin. Values shown represent the mean + SE of triplicates.

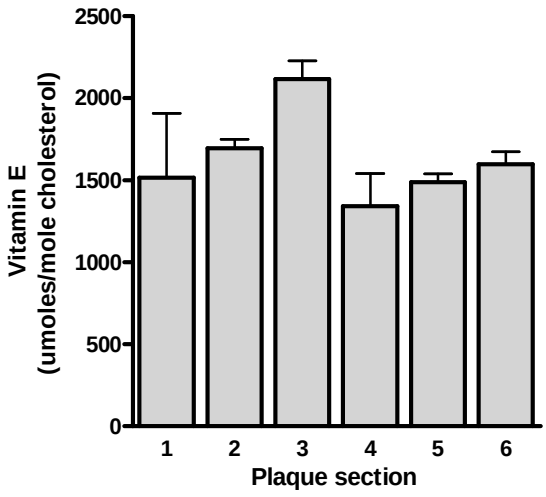


**Figure 34: Vitamin E concentration in six sections of plaque E.**

Individual sections of plaque D were analysed for their vitamin E content using HPLC. Values shown represent the mean + SE of triplicates.

**Vitamin E**

The first bifurcation section, 3, contains significantly higher ($p<0.05$) levels of vitamin E compared to the secondary branch (section 4) (Figure 34). Otherwise there is no significant variation across the length of the plaque, and no pattern is apparent either in regards to localisation or the observed calcium content of the sections.

**Markers of oxidation**

The average DOPA content of plaque E is 84 ± 16 nmoles per gram of protein (Figure 35A). Carbonyl concentration across the entire plaque averaged to 3.0 ± 0.5 µmoles per gram of protein (Figure 35B). Due to large variation within the section's replicates neither DOPA nor carbonyl content of plaque E showed any significant variation between the sections.

The leading edge of plaque E shows significantly greater TBARS content ($p<0.05$) then that in the immediately adjacent section and the distal ends of both branches (Figure 35C). Similar levels to those in section 1 are also present in the gruelly sections of the bifurcation (sections 3 and 5).

Dityrosine was not present in any of the sections of plaque E within the detection limits of the assay (data not shown).

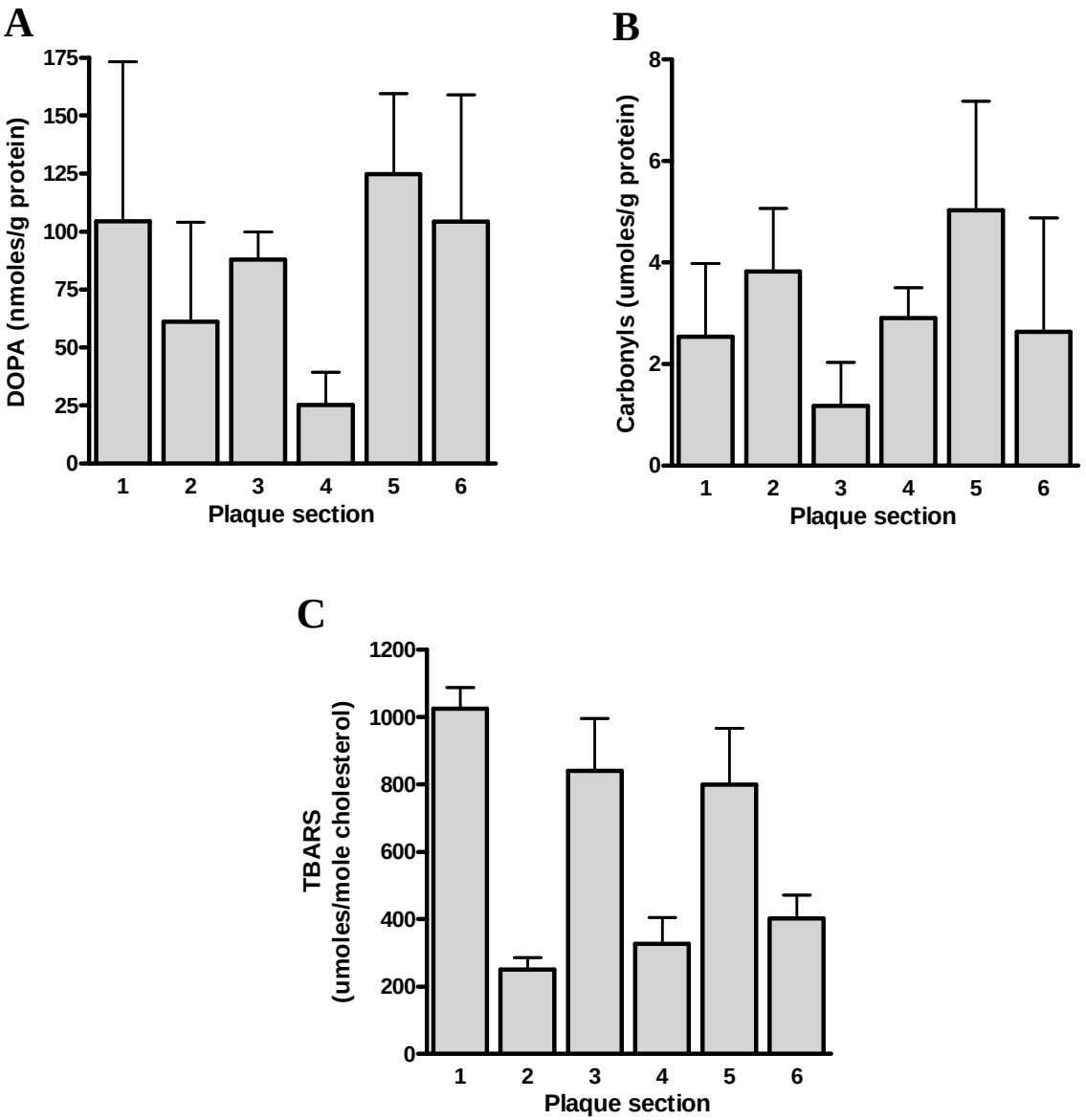


**Figure 35: Markers of oxidation in six sections of plaque E.**

Individual sections of plaque E were analysed for their content of: DOPA (A), carbonyl (B) and TBARS (C). Values shown represent the mean + SE of triplicates.

## Plaque F – GP050407

Approximately 32 mm of plaque material was removed from the left common carotid artery of patient F (Figure 36). It was dissected into six segments ranging from 0.04-0.11 g in weight. The plaque was classified as thrombosed as blood clotting was evident throughout the plaque especially in sections 5 and 6. For purposes of between plaque comparisons, sections 1 and 2 were assigned as pre-bifurcation, 3-5 as bifurcation and section 6 as post-bifurcation.

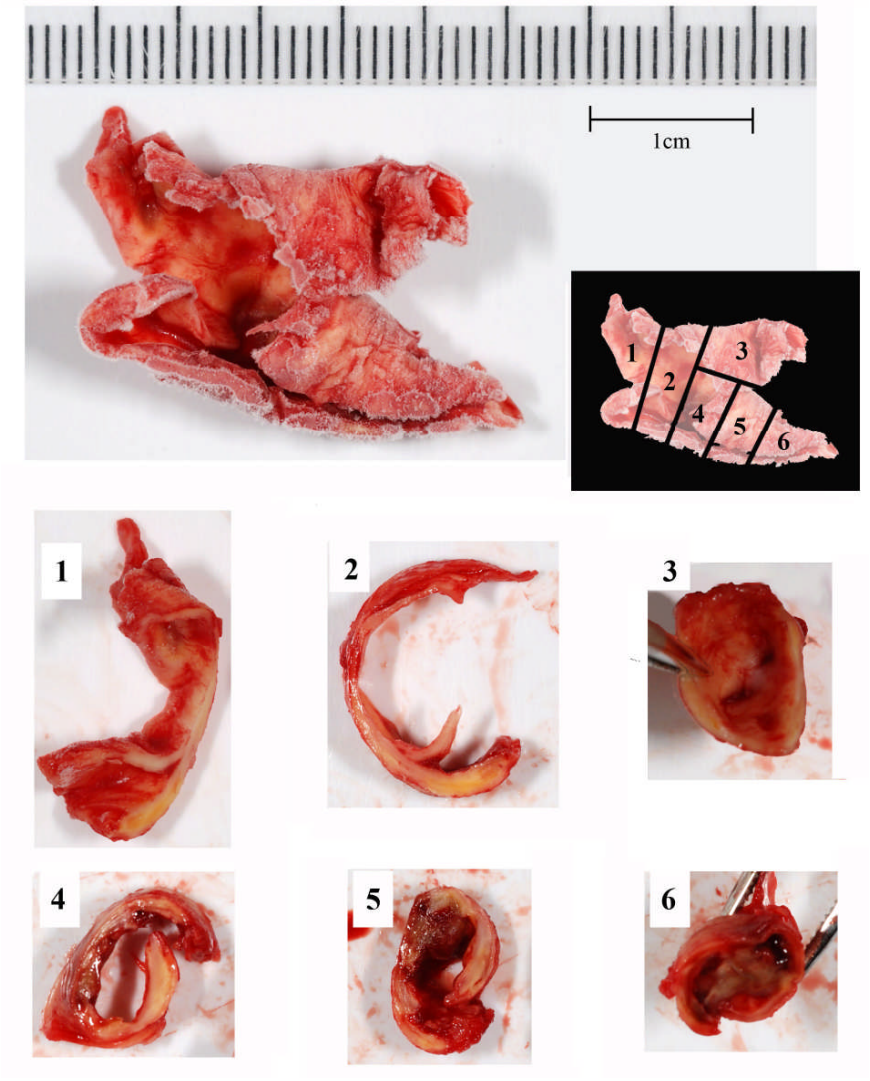


**Figure 36: Plaque F - Cross sections of the atheromatous material removed from the carotid artery of patient F.**

The plaque was separated and removed from the left common carotid artery of patient F. It was dissected into six segments labelled sections 1-6 as shown. Sections 1-2 were assigned as pre-bifurcation, section 3-5 as bifurcation and section 6 as post-bifurcation.

**Protein and cholesterol**

Protein concentration of plaque F peaks in the distal sections of the primary branch around and after the bifurcation ($p<0.05$) (Figure 37A). The preceding sections show no significant variations.

The leading edge and distal section of the secondary branch have significantly lower ($p<0.01$) cholesterol contents compared with the other sections, which show almost twice as much cholesterol per gram of plaque (Figure 37B).

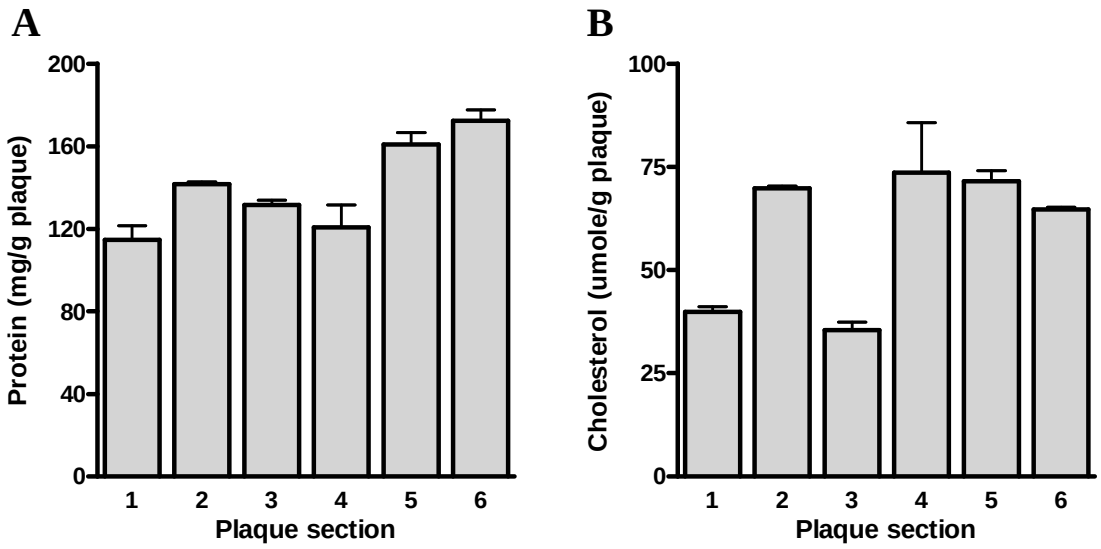


**Figure 37: Protein and cholesterol concentrations in six sections of plaque F.**

Protein content (A) was analysed in individual sections using a BCA protein determination kit. Cholesterol content (B) was determined in each section by spectrophotometer. Values shown represent the mean + SE of triplicates.

**Neopterin**

Neopterin for plaque F was measured using the acetonitrile protein precipitation method. The pre-bifurcation sections and the secondary branch contain lower neopterin concentrations with the lowest concentration occurring in the leading edge (Figure 38). Neopterin concentration is highest in section 4 then drops moving down the primary branch though not dropping as low as levels in the earlier sections ($p<0.05$).

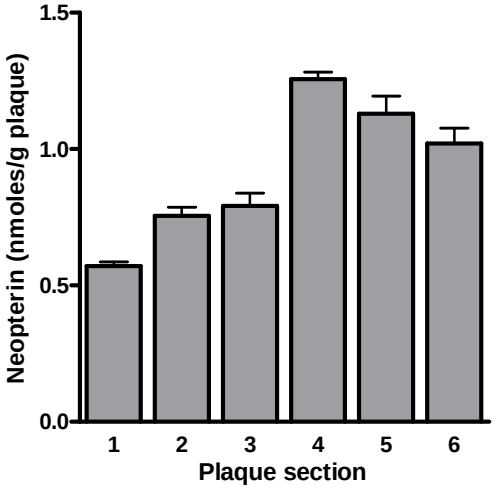


**Figure 38: Neopterin concentration in six sections of plaque F.**

Individual sections of plaque F were treated with ACN to precipitate proteins prior to HPLC analysis for neopterin. Values shown represent the mean + SE of triplicates.

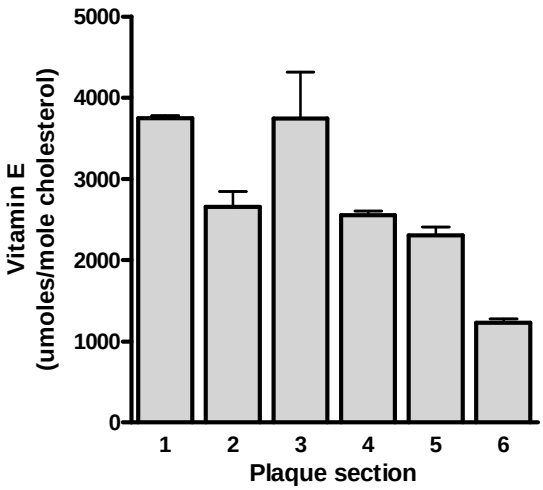


**Figure 39: Vitamin E in six sections of plaque F.**

Individual sections of plaque E were analysed for their vitamin E content using HPLC. Values shown represent the mean + SE of triplicates.

**Vitamin E**

Vitamin E shows the lowest concentration per mole of cholesterol in the distal end of the primary branch ($p<0.05$) (Figure 39). The peaks in sections 1 and 3 correspond to areas of low cholesterol levels; however they are not significantly different from sections 2 and 4 which border them.

**Markers of oxidation**

The average concentration of DOPA across plaque F was 72 ± 5 nmoles per gram of protein (Figure 40A). Dityrosine gave an average of 67 ± 7 nmoles per gram of protein (Figure 40B). No significant variation between sections is shown by either of the oxidative markers.

The majority of sections in plaque F showed no significant variation in carbonyl content; section 5 being the exception with a significantly greater ($p<0.05$) concentration of carbonyls per gram of protein (Figure 40C).

Significant differences in TBARS content cannot be attributed between the sections of plaque F apart from sections 3 and 4 which represent lowest and highest ($p<0,05$) TBARS concentration per gram of cholesterol respectively (Figure 40D).

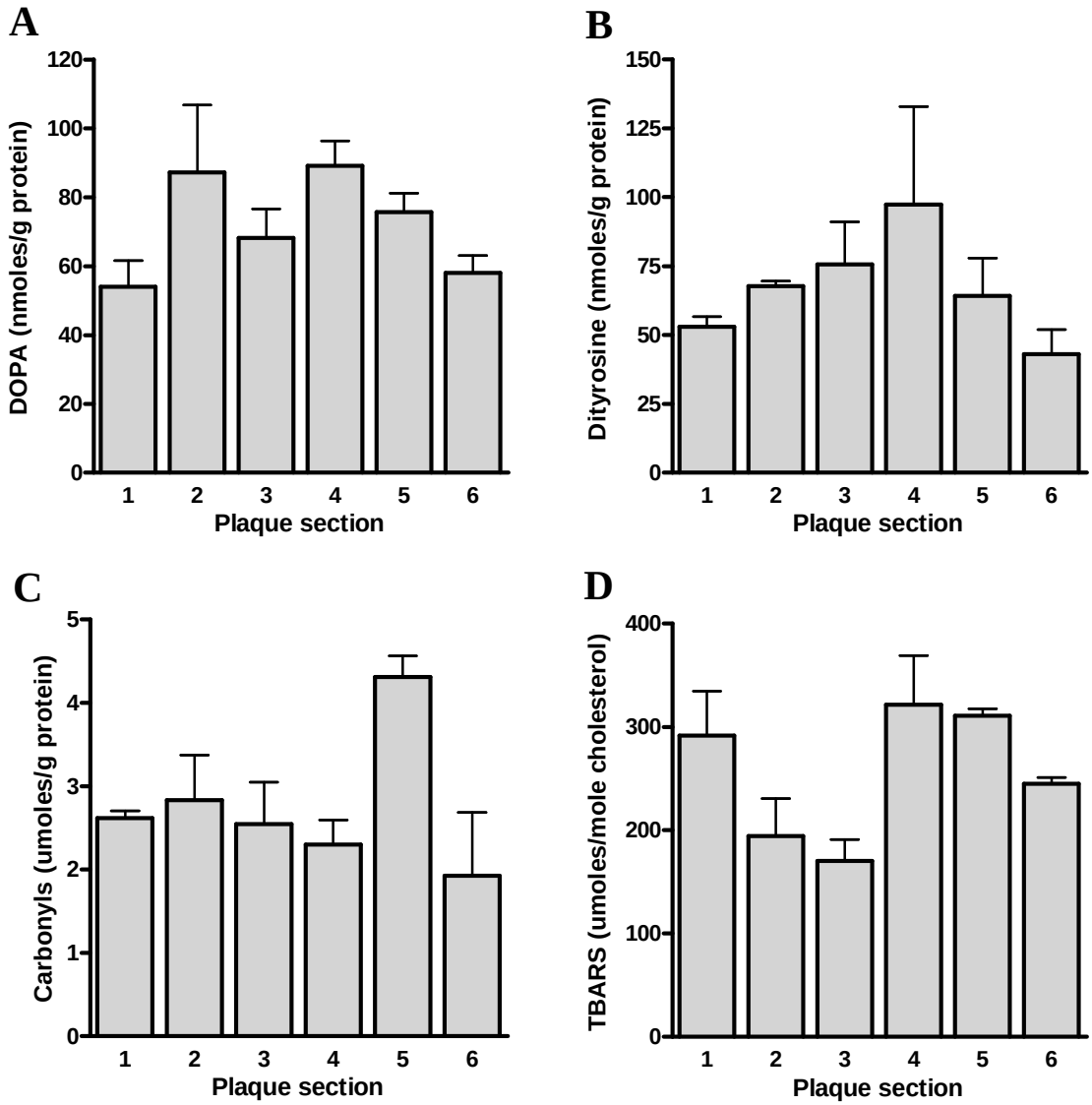


**Figure 40: Markers of oxidation for six sections of plaque F.**

Individual sections of plaque F were analysed for their content of: DOPA (A), dityrosine (B), carbonyl (C) and TBARS (D). Values shown represent the mean + SE of triplicates.

## Correlations between markers of oxidation and inflammation for individual and combined plaques

Both the combined plaque data and the individual plaque data was assessed for correlations. Significant relationships are outlined in table 3. Data was expressed per gram of plaque for the analysis.

Relationships for neopterin were assessed in two separate groups depending on the method used for protein precipitation prior to measurement (TCA or Acetonitrile). This is due to the profound difference in neopterin levels produced by this factor that may mask any relationships between other markers. The method involved in the relationship is marked in brackets, with the abbreviation ACN used for acetonitrile.

Of the significant correlations seen in the combined data set, only five were found to also be significant in an individual plaque: dityrosine vs. cholesterol and carbonyl vs. vitamin E in plaque B; carbonyls vs. protein and neopterin (ACN) vs. carbonyls in plaque E; and TBARS vs. vitamin E in plaque C.

Only two correlations were present in more then one plaque: vitamin E vs. cholesterol was significant in plaques A and E; and neopterin (ACN) vs. protein was significant in plaques C and E. Despite these relationships appearing in two plaques; the relationship did not hold in the combined plaque data.

Plaque D was the only plaque to demonstrate any significant negative correlations (Table 3) and only one negative correlation appears in the combined plaque data, DOPA vs. vitamin E, which does not correspond with the associations found in plaque D.

**Table 3: Correlations between markers of oxidation and inflammation in sectioned plaques.**

Plaque sections were analysed for the content of protein, cholesterol, neopterin, vitamin E, DOPA, dityrosine, carbonyls and TBARS. Relationships between these parameters were assessed for the combined data set and for the individual plaques. Data was expressed per gram of plaque for the analysis. Significant associations are listed and the level of significance is indicated by * for $p<0.05$; ** for $p<0.01$; or *** for $p<0.001$. Relationships found in individual plaques that correspond to combined plaque data are noted in brackets beside the combined plaque correlation. Relationships present in more then one plaque are marked with †. Plaque classifications are given beside the plaque.

| Correlation | r (Pearson) | p value | | N |
|---|---|---|---|---|
| **Combined Plaque Data** | | | | |
| DOPA vs. Vitamin E | -0.292 | 0.033 | (*) | 53 |
| DOPA vs. Dityrosine | 0.330 | 0.016 | (*) | 53 |
| Dityrosine vs. Cholesterol (*B*) | 0.477 | 0.000 | (***) | 53 |
| Carbonyl vs. Protein (*E*) | 0.346 | 0.011 | (*) | 53 |
| Carbonyl vs. Vitamin E (*B*) | 0.430 | 0.001 | (**) | 53 |
| TBARS vs. Protein | 0.485 | 0.000 | (***) | 53 |
| TBARS vs. Vitamin E (*C*) | 0.286 | 0.038 | (*) | 53 |
| Vitamin E vs. Protein | 0.356 | 0.008 | (**) | 53 |
| Neopterin (ACN) vs. Carbonyl (*E*) | 0.465 | 0.019 | (*) | 25 |
| **Plaque A - Neither** | | | | |
| Dityrosine vs. Protein | 0.801 | 0.017 | (*) | 8 |
| Vitamin E vs. Cholesterol † | 0.970 | 0.000 | (***) | 8 |
| **Plaque B - Thrombosed** | | | | |
| Dityrosine vs. Cholesterol | 0.766 | 0.044 | (*) | 7 |
| Carbonyl vs. Vitamin E | 0.790 | 0.034 | (*) | 7 |
| **Plaque C - Calcified** | | | | |
| Neopterin (ACN) vs. Protein † | 0.853 | 0.015 | (*) | 7 |
| Vitamin E vs. TBARS | 0.784 | 0.037 | (*) | 7 |
| **Plaque D - Thrombosed** | | | | |
| Protein vs. Cholesterol | -0.866 | 0.026 | (*) | 6 |
| Neopterin (ACN) vs. Dityrosine | -0.822 | 0.045 | (*) | 6 |
| Carbonyls vs. Cholesterol | -0.823 | 0.044 | (*) | 6 |
| Carbonyls vs. TBARS | 0.851 | 0.032 | (*) | 6 |
| **Plaque E - Calcified** | | | | |
| Neopterin (ACN) vs. Protein † | 0.971 | 0.001 | (**) | 6 |
| Neopterin (ACN) vs. Carbonyls | 0.866 | 0.026 | (*) | 6 |
| Carbonyls vs. Protein | 0.881 | 0.021 | (*) | 6 |
| Vitamin E vs. Cholesterol † | 0.932 | 0.007 | (**) | 6 |
| **Plaque F - Thrombosed** | | | | |
| DOPA vs. Cholesterol | 0.825 | 0.043 | (*) | 6 |
| TBARS vs. Cholesterol | 0.855 | 0.030 | (*) | 6 |
| TBARS vs. Neopterin (ACN) | 0.827 | 0.042 | (*) | 6 |

## Analysis of Variance for markers of oxidation and inflammation for combined plaque data

Factorial ANOVA was performed on the pooled plaque data to establish the effects of localisation zone within the plaque (pre-, post- or bifurcation) and classification of the plaque (heavily calcified or thrombosed) on markers of oxidation and inflammation. In the case of neopterin method of measurement (Acetonitrile or TCA) was also assessed.

As with the individual plaque data; DOPA, dityrosine and carbonyls were analysed per gram of protein and vitamin E and TBARS per gram of cholesterol. Prior to ANOVA samples were assessed for homogeneity of variances and normality; if data did not fit these assumptions it was transformed accordingly (logarithmic transformation). Only variables that showed significant effects are given.

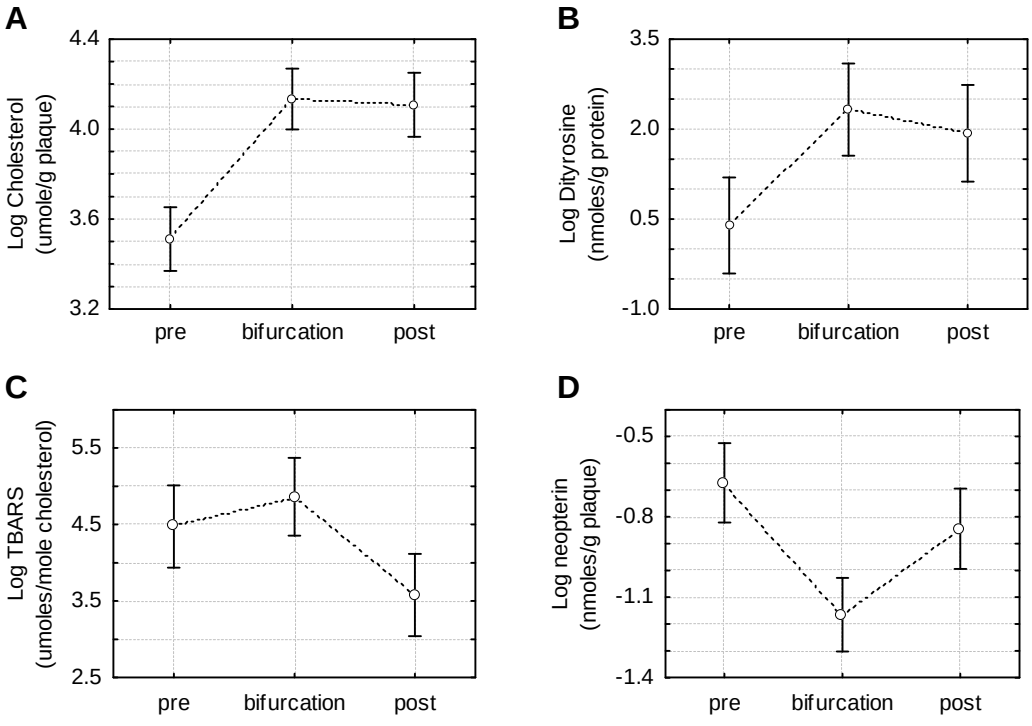


**Figure 41: Variance in oxidative markers due to localisation within the plaque.**

ANOVA results for cholesterol (A), dityrosine (B), TBARS (C) and neopterin (D); LS mean by zone (pre-, post- and bifurcation). Vertical bars denote 0.95 confidence intervals.

**Variance due to localisation in pre-, post- and bifurcation zones**

Cholesterol showed lower concentrations in the pre-bifurcation zone then those in the bifurcation and post-bifurcation zones ($p<0.001$) (Figure 41A). Levels of dityrosine in plaques were lower in the pre-bifurcation zone then in the bifurcation zone ($p<0.05$) (Figure 41B). TBARS concentrations were higher in the bifurcation zone of plaques then in the post-bifurcation zone ($p<0.001$) (Figure 41C). Neopterin concentrations were lowest in the bifurcation zone of plaques ($p<0.05$) (Figure 41D).

**Variance due to classification as containing thrombosis, heavily calcified or neither**

Plaques classified as containing thrombosis showed higher protein content then both the heavily calcified plaque and plaques classified as neither ($p<0.001$) (Figure 42A). Cholesterol concentration of thrombosed plaques was higher then the

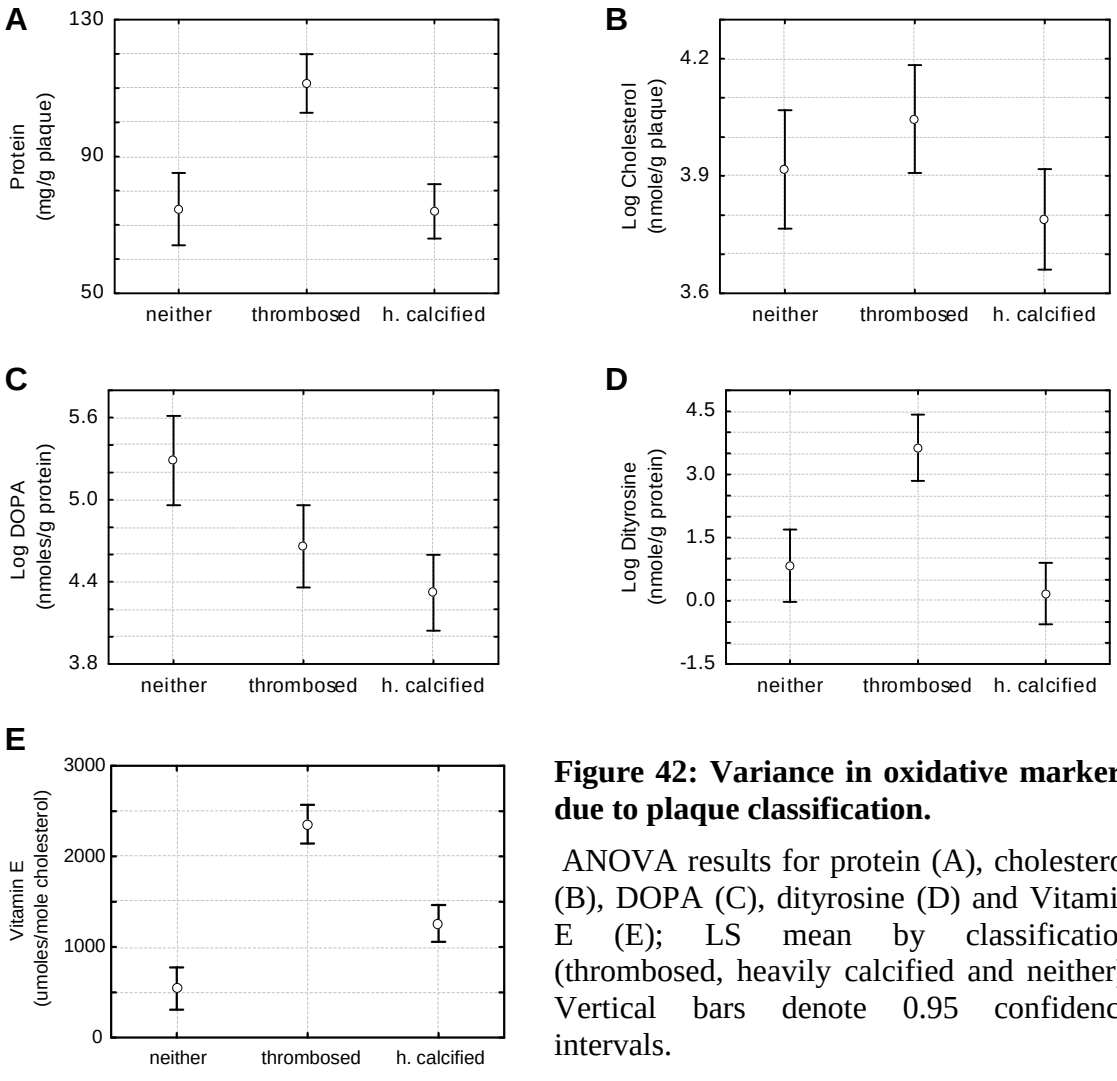


**Figure 42: Variance in oxidative markers due to plaque classification.**

ANOVA results for protein (A), cholesterol (B), DOPA (C), dityrosine (D) and Vitamin E (E); LS mean by classification (thrombosed, heavily calcified and neither). Vertical bars denote 0.95 confidence intervals.

concentration found in heavily calcified plaques ($p<0.05$) (Figure 42B). DOPA content is lower in both the thrombosed ($p<0.05$) and heavily calcified ($p<0.001$) plaques then in the plaques classified as neither (Figure 42C). Plaques with evidence of thrombosis showed higher levels of dityrosine then both the heavily calcified plaques and those plaques that were neither thrombosed or calcified heavily ($p<0.001$) (Figure 42D). Vitamin E content differed between each of the plaque groups with thrombosed plaques showing the greatest amount ($p<0.001$), followed by heavily calcified plaques and plaques classified as neither showing the least ($p<0.001$) (Figure 42E).

**Variance due to interactions between zone and classification**

Along with a significant variation due to zone and classification individually cholesterol also showed a significant interaction between the two factors ($p<0.001$) with plaques classified as neither thrombosed nor heavily calcified showing a spike in cholesterol in the bifurcation zone in relation to pre- and post- zones and the other classifications (Figure 43A).

Carbonyls, which failed to show significant variation with the individual factors, did show a significant interaction between zone and classification ($p<0.01$). The plaques classified as neither thrombosed nor heavily calcified deviated from the general trend by showing lower carbonyl concentrations in the pre bifurcation zone then in the post- and bifurcation zones and in relation to the other classifications (Figure 43B).

While the single factor ANOVA demonstrated the thrombosed plaques to be lower in DOPA content then plaques in the ‘neither’ classification, multi-factor ANOVA demonstrates that in fact the thrombosed plaques only have significantly lower DOPA content in the post-bifurcation zone and not across the entire plaque ($p<0.05$) (Figure 43C).

ANOVA of dityrosine for classification alone showed thrombosed plaques to be significantly higher in dityrosine then the other plaques; however, inclusion of zone introduces a significant interaction ($p<0.001$). The interaction factor shows that thrombosed plaques have significantly higher dityrosine then ‘neither’ plaques in the post-bifurcation zone only. Conversely, heavily calcified plaques have significantly lower dityrosine then thrombosed plaques in the pre- and bifurcation zones but similar levels in the post-bifurcation zone.

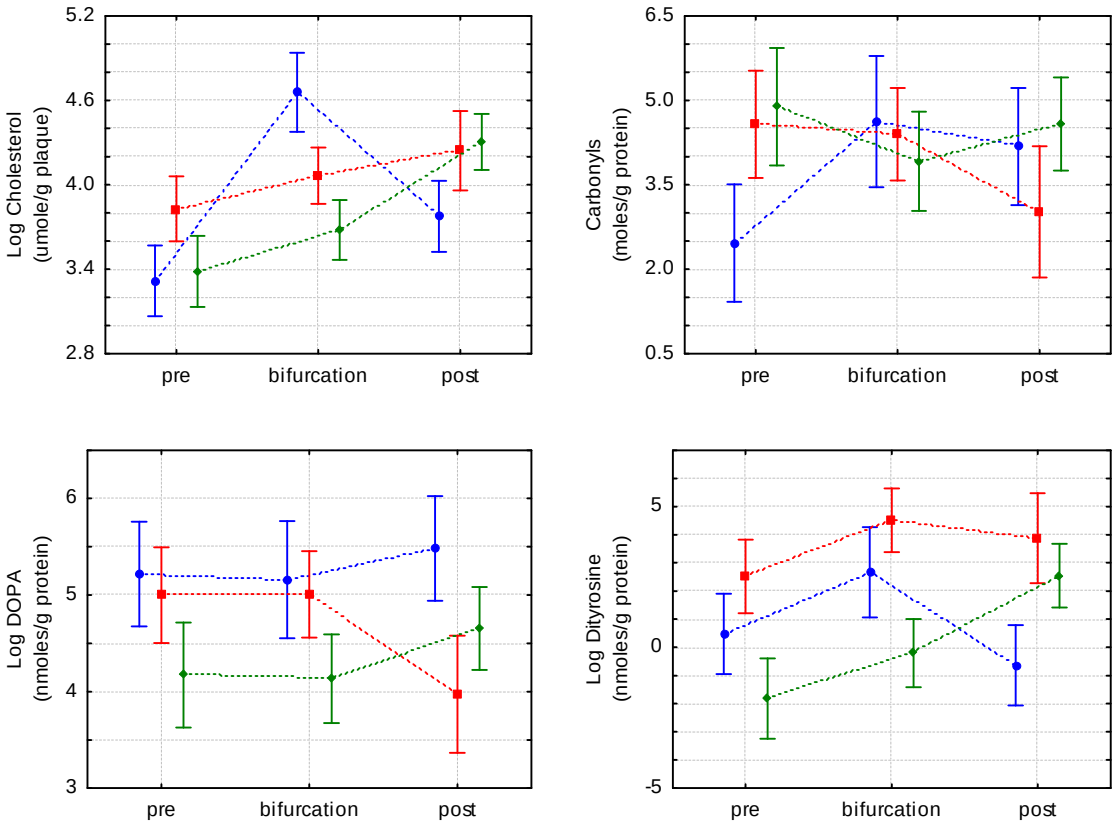


**Figure 43: Variance in oxidative markers due to interactions between plaque classification and zone.**

Factorial ANOVA results for cholesterol (A), carbonyls (B), DOPA (C), and dityrosine (D); LS mean for zone (pre-, post- and bifurcation) vs. classification (thrombosed, heavily calcified, and neither). Vertical bars denote 0.95 confidence intervals.

### Variance in neopterin concentration of plaques due to the method of measurement

The method of protein precipitation prior to neopterin measurement produces a significant difference in the value of neopterin obtained ($p<0.001$). As observed with plasma samples in the neopterin analysis development, measurement of plaque samples with acetonitrile as the precipitation reagent demonstrated higher values of neopterin then that produced with a TCA precipitant (Figure 44A).

A significant interaction factor between method of measurement and zone was demonstrated ($p<0.001$). It showed the pre-bifurcation zone to have higher neopterin levels then the corresponding bifurcation and post-bifurcation zones, but only when measured with the TCA precipitation method, as this trend was not present in plaques measured by acetonitrile precipitation (Figure 44B).

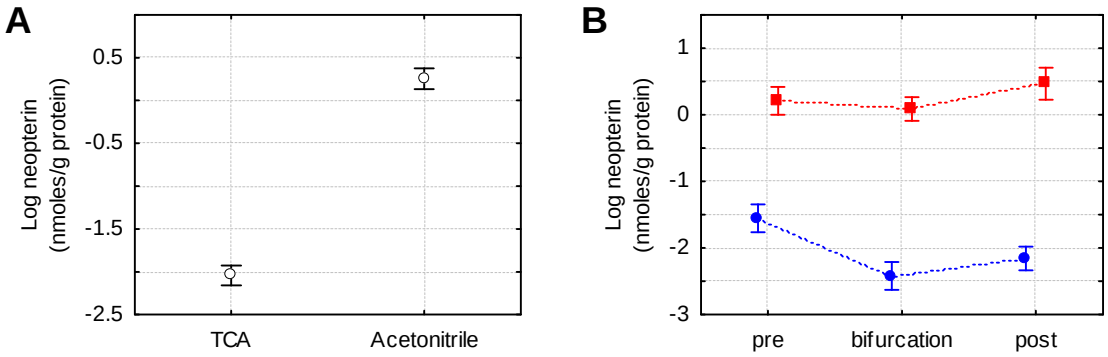


**Figure 44: Variance in neopterin due to method of measurement.**

ANOVA results for neopterin: (A) LS mean by method of measurement (TCA and Acetonitrile) and (B) LS mean by method of measurement (TCA and Acetonitrile ) vs. zone (pre-, post- and bifurcation). Vertical bars denote 0.95 confidence intervals.

# Discussion

## Neopterin Analysis Development

Initial research was focused on improving detection of neopterin and 7,8-dihydroneopterin (78NP) in plasma. Previous analysis of neopterin in the laboratory had been performed with trichloroacetic acid (TCA) as the protein precipitation reagent prior to HPLC. However; acidic conditions have been previously reported to cause significant oxidation of 78NP to neopterin (Werner et al. 1987). With TCA we observed up to 60% of 78NP being oxidised to neopterin (Figure 6). Similar problems were also encountered using perchloric acid. The effect of this oxidation would not be apparent when dealing with neopterin samples alone, but would cause a substantial underestimate of 78NP concentrations and a slight additional overestimate of neopterin when working with biological samples. Ascorbate was found to reduce this oxidation but was not completely effective (Figure 6). This effect of ascorbate has been described previously (Werner et al. 1987). Therefore acetonitrile was examined as an alternative to acid precipitation of proteins. We found that 78NP was not oxidised by treatment with acetonitrile making it a more suitable reagent for protein precipitation.

The use of acetonitrile, as the protein precipitating reagent in plasma prior to HPLC measurement of neopterin gave several additional advantages over TCA. Acetonitrile not only improved the resolution of peaks and the signal to noise ratio in HPLC chromatograms (Figure 7) but also gave considerably higher neopterin concentrations (Figure 10). This increase in neopterin concentration with acetonitrile was also apparent in measurement of plaque samples where a significant difference in neopterin concentration was seen between the acetonitrile and TCA treated groups (Figure 44).

The variation between the acetonitrile and TCA treated plasma samples in neopterin and total neopterin levels can not be fully explained by the acid-induced oxidation of 78NP to neopterin. If this was the case only total neopterin concentrations would be expected to increase; instead the levels of both total neopterin and neopterin increased dramatically with the acetonitrile treatment. This increase in neopterin with acetonitrile treatment was not observed using protein free buffers which suggests the effect is due to the actual precipitation of protein. The most plausible explanation being

that in addition to the acid oxidation, a varying level of pterin is co-precipitating with the serum proteins and is lost from the sample when treated with TCA.

TCA treatment shows a picture of healthy subjects having the majority of the pterin as 78NP (75%) (Figure 10) which is close to the published 2:1 ratio (Wachter et al. 1992). In the septicemia patients, this ratio is reversed where the majority of the pterin is neopterin (83%). This does agree with the hypothesis that 78NP can be oxidised to neopterin by oxidants produced during inflammation. However, the TCA data does not show a significant overall increase in total pterin when comparing the healthy controls and the septicemia patients (Figure 10). This is not consistent with what is known about pterin release from macrophages during the immune response (Huber et al. 1984, Wachter et al. 1989). The acetonitrile data shows the overall level of pterins in the plasma increasing 203% with the level of 78NP increasing from 19% to 28% which would be consistent with increased production of 78NP by macrophage cells. This is especially relevant if one of the roles of 78NP is to provide protection to cells and biomolecules(Gieseg et al. 1995, Gieseg et al. 2001b, Mori et al. 1996). The overall increase in neopterin/78NP is also consistent with the hypothesis that neopterin increases the potency of various cytotoxic agents (Enzinger et al. 2002b, Murr et al. 1994, Oettl et al. 1999).

## Oxidative Stress and Antioxidant Activity within the Atherosclerotic Plaque

Atherosclerosis is a very complex disease with many contributing factors. The advanced plaques removed in endarectomy surgery represent a lifetime of lipid deposition, low-level inflammation and oxidative stress. At this late stage of the disease the biochemical morphology of plaques has diversified such that subclassifications are required to describe the composition. This diversity is evident in the lack of consistent trends found between the various markers measured. Significant correlations were rarely seen in more then one plaque and trends found in the combined data set generally did not hold true in individual plaques. It is therefore difficult to comment on any relationship between the various markers.

It is perhaps of more interest to note those things that one might have expected to correlate which did not. There is little correlation between the various markers of oxidation (Table 3); there is a relationship between DOPA and dityrosine across the combined plaque data and in one plaque a correlation between TBARS and carbonyls; otherwise there is no consistent pattern in oxidation levels. This suggests that there is unlikely to be any oxidation "hotspots" within the plaque. An alternate explanation could be the extensive remodelling that occurs in advanced plaque due to damage to structural components, incorporation of thrombi and repair mechanisms (Stary et al. 1995). This remodelling at late stages of plaque development could disrupt patterns of high localised oxidative incidence.

Vitamin E and cholesterol correlated strongly in two plaques (Table 3), which might be expected as vitamin E is lipid soluble; however, this relationship was not seen in the combined plaque data. This suggests that, in the majority of the plaques tested, vitamin E concentration is affected by something other than cholesterol content. It might be hypothesised that the vitamin E is acting as an antioxidant and being depleted in this manner. This would suggest a high radical flux and thus higher oxidation levels. This may be illustrated in the negative correlation between vitamin E and DOPA; however, measurement of lipid oxidation by TBARS shows a positive correlation with vitamin E. This could imply a pro-oxidative role of vitamin E, particularly in areas of low radical flux. Further investigation of the possibility of TMP occurring within atherosclerotic plaque would require the quantification of co-antioxidants such as ascorbate and ubiquinol-10. If low concentrations of these co-oxidants were to correlate with high incidences of TBARS/Vitamin E it would strongly support the theory of TMP occurring locally within the atherosclerotic plaque. Further to this, in areas of the plaque were co-oxidants have not been depleted, vitamin E would be expected to still be functioning as antioxidant.

Neopterin and protein correlated strongly in the two highly calcified plaques (Table 3). Protein and neopterin both tended to be lower in the sections where calcification was most marked. These lower values of neopterin and protein do not manifest as an overall decrease in comparison to non-calcified plaques so it is likely that these correlations are due to the calcium adding mass to the plaque sections without directly affecting the quantity of protein present. A quantifiable measure of calcium would be required to confirm this.

The potential for neopterin to influence calcium deposition within the atherosclerotic plaque has been speculated on (Gieseg et al. 2007). Little research has been done in this area but both neopterin and 78NP have been shown to increase the intracellular calcium concentration of monocytes from the human THP-1 cell line (Woll et al. 1993). This increase is dependant upon the presence of extracellular calcium in the medium. It is possible then that less calcification is seen in areas of high neopterin as this induces uptake of calcium into cells.

Though there is no considerable difference in protein concentration between calcified and non-calcified plaques; thrombosed plaques did show markedly higher protein levels then those which are not thrombosed (Figure 42A). This is most likely due to the incorporation of the highly proteinacious material, predominately platelets and red blood cells, that the thrombus consists of. The higher values of dityrosine (Figure 42D) and vitamin E (Figure 42E) seen in thrombosed plaques may also derive from this influx of red blood cells. It has been demonstrated that dityrosine can be formed on intact red blood cells during oxidant exposure *in vitro* (Giulivi and Davies 1993). It has also been estimated that 3% of red blood cells undergo autooxidation daily, generating superoxide that dismutases to hydrogen peroxide (Misra and Fridovich 1972), which would be sufficient to produce significant amounts of dityrosine in red blood cells. Therefore it is possible that inclusion of oxidised red blood cells can account for increased levels of dityrosine within the thrombosed atherosclerotic plaques. Similarly there is a relatively high content of vitamin E within the membrane of red blood cells (Simon et al. 1997) and this could contribute to higher vitamin E concentrations seen in plaques with incorporated thrombis.

Separation of the plaques into pre-, post-, and bifurcation areas did produce some trends. The lower cholesterol content seen in pre-bifurcation sections (Figure 41A) could be consistent with the proximal end of the plaque being the “leading edge” of new plaque growth as this area of comparatively new plaque tissue would have had less time to accumulate LDL. Similarly the trend towards lower dityrosine content in the pre-bifurcation area of plaques (Figure 41B) could be indicative of “younger” tissue. Alternatively, oscillatory and low shear stress typically occur at, and downstream of, branch points in the artery (Chatzizisis et al. 2007). Low endothelial shear stress leads to the amplified uptake, synthesis and permeability of LDL while at the same time promoting oxidative stress and inflammation(Chatzizisis et al. 2007). This could explain

the higher levels of cholesterol and dityrosine seen in the bifurcation and post-bifurcation zones, which are the areas subjected to disrupted and low shear stress.

The trend of low dityrosine in the pre-bifurcation zone is not mirrored in the other markers of protein oxidation which showed no significant difference between zones. TBARS measurements showed an opposing trend of lower lipid oxidation in the post-bifurcation section (Figure 41C). The lack of a similar trend to that of dityrosine in other protein oxidation markers could be due to a lack of precision of the assays, preventing such comparisons between zones being made. The opposing trend in TBARS is more difficult to justify in the context of this theory. It could be that TBARS is more strongly influenced by factors other then zoning.

Neopterin concentrations showed a trend of lower levels in the bifurcation zone (Figure 41D) which could be accounted for by the presence of more activated macrophages in the peripheral sections which have not yet become part of the necrotic core. There is a tendency towards higher neopterin levels in the pre-bifurcation zone which is consistent with the concept of this area being the newest area of plaque growth with a greater content of activated macrophages that have not yet succumbed to the toxic environment of the atherosclerotic plaque. However, it should be noted that when the data is separated according to the method used for neopterin measurement the trend is only apparent in those plaques measured by the TCA method (Figure 44B). It may be that this effect is related to the generally smaller mass of sections in the pre-bifurcation sections. This causes the homogenate of these sections to be more dilute. It is possible that this dilution causes the neopterin in these sections to be less affected by the protein interaction effects described earlier in the analysis development section of the discussion. This would cause higher readings then that obtained in less dilute sections.

With the presence of activated macrophages it might be expected to find 78NP; however, no significant quantity of 78NP was found within the atherosclerotic plaques (results not shown). There are several possible explanations for its absence. It has been suggested that 78NP is produced to protect macrophages from the oxidative environment that they themselves generate (Gieseg et al. 2007). It may be that at this late stage of plaque development the macrophages defensives have been exhausted and with the depletion of 78NP also comes macrophage death. In order to assess this theory the 78NP content of plaques in earlier stages of development would need to be measured. Alternatively, it could be that the 78NP content of the plaques is oxidised during the

homogenisation process. 78NP is relatively labile and the contents of atherosclerotic plaques has been demonstrated to be highly oxidative (Smith et al. 1992); it may be possible that the BHT and EDTA added to augment unwanted oxidative reactions occurring during the experimental process was not sufficient to counteract this highly oxidative environment.

While it may be tempting to use neopterin as an estimate of 78NP content, it is not the only product of 78NP oxidation. The only mechanism for the *in vivo* production of neopterin from 78NP, at present, is the reaction with hypochlorite. The scavenging of hydrogen peroxide, superoxide and peroxyl radicals by 78NP produces 7,8-dihydroxanthopterin (Duggan et al. 2002) (Figure 5). Unfortunately, 7,8-dihydroxanthopterin is not fluorescent and is subsequently difficult to detect at low µM levels in plaque. Thus any approximation made on 78NP content based upon its oxidative products which fails to take into account 7,8-dihydroxanthopterin would be a substantial underestimate.

While localisation of antioxidants and oxidative markers in sectioned plaques has not been performed prior to this study, measurement of some of the markers of oxidation and Vitamin E in plaque samples has been done previously. Vitamin E content of the intima of advanced plaque has been measured resulting with an average concentration (± SD) of 6.3 ± 4.8 mmoles/mole free cholesterol (Suarna et al. 1995). Our average across all plaques of 1.5 ± 1.0 mmoles/mole of cholesterol is comparable, if somewhat lower. This is most likely due to our use of all plaque material rather then restricting our measurements to the intima only. TBARS have been previously measured in plaque samples from carotid enderactomy surgery where the average TBARS value (± SD) was 36.4 ± 4.2 nmoles/g tissue by butanol abstraction and absorption methods (Nishi et al. 2002). Our results were considerably lower; 7.9 ± 5.8 nmoles/g plaque across all samples using fluorescence detection with HPLC. The butanol/absorption method is known to have problems with over-reading samples, due to the volatility of the solvents during abstraction and the lack of specificity in the absorption readings (Halliwell and Gutteridge 1999). These problems are overcome using HPLC fluorescence detection so it is likely that our results are a more accurate measure of lipid peroxidation. DOPA and dityrosine levels have been assessed in intima tissue from advanced plaques with mean values (± SD) of 14.26 ± 3.80 and 4.75 ± 5.17 nmoles/g of tissue respectively (Fu et al.

1998). We obtained remarkably similar means values (± SD) of 13.5 ± 9.7 nmoles/g of plaque for DOPA and 4.6 ± 7.2 nmoles/g of plaque for dityrosine.

This study clearly demonstrated that markers of oxidative stress and inflammation do vary across the length of an atherosclerotic plaque. This variation could allow for localized incidences of high and low radical flux and microenvironments of depleted antioxidants or areas in which the prooxidative actions of molecular components are favoured. While consistent patterns between the markers were not readily evident, some patterns may emerge with a larger data set where the plaques can be more effectively grouped according to their compositional characteristics. There is no doubt that the atherosclerotic plaque is a highly oxidative environment however the nature of this environment varies within the plaque and between plaques which reflects upon the complexity of the disease. Some variations can be related to the specific spatial localization within the plaque in relation to the shear stress variations in the blood flow; further investigations into the biochemical differences between these areas may provide a better understanding of the growth and development of the atherosclerotic plaque.

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# Appendix I

**Table 4: Protein, cholesterol, neopterin and vitamin E concentrations for plaque sections.**

| Plaque | Section | Protein (mg/g plaque) | | Cholesterol (umoles/g plaque) | | Neopterin (nmoles/g plaque) | | Vitamin E (nmoles/g plaque) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| IH120704 A | 1 | 98.13 | 2.23 | 57.09 | 2.20 | 0.14 | 0.02 | 29.94 | 3.19 |
| | 2 | 108.41 | 4.61 | 104.94 | 8.40 | 0.10 | 0.00 | 48.82 | 0.54 |
| | 3 | 80.51 | 0.30 | 128.22 | 6.98 | 0.07 | 0.00 | 61.95 | 0.29 |
| | 4 | 54.05 | 3.56 | 121.83 | 6.84 | 0.09 | 0.00 | 75.55 | 2.65 |
| | 5 | 80.73 | 0.40 | 92.66 | 9.80 | 0.10 | 0.01 | 53.44 | 0.29 |
| | 6 | 57.40 | 1.71 | 33.74 | 1.58 | 0.09 | 0.00 | 18.47 | 0.78 |
| | 7 | 36.91 | 0.57 | 23.07 | 1.17 | 0.07 | 0.00 | 8.84 | 0.18 |
| | 8 | 46.27 | 0.64 | 23.86 | 1.37 | 0.03 | 0.00 | 22.41 | 0.63 |
| HW290304 B | 1 | 93.89 | 4.11 | 25.54 | 5.37 | 0.31 | 0.01 | 11.90 | 1.24 |
| | 2 | 80.32 | 0.69 | 88.78 | 2.54 | 0.47 | 0.01 | 57.66 | 3.11 |
| | 3 | 82.77 | 1.37 | 93.48 | 3.54 | 0.10 | 0.00 | 37.35 | 1.58 |
| | 4 | 68.33 | 3.52 | 116.46 | 13.05 | 0.17 | 0.02 | 5.75 | 0.38 |
| | 5 | 84.83 | 4.08 | 60.19 | 3.85 | 0.08 | 0.01 | 17.99 | 0.41 |
| | 6 | 93.27 | 3.71 | 91.27 | 4.48 | 0.09 | 0.01 | 13.74 | 0.02 |
| | 7 | 128.12 | 1.64 | 90.52 | 2.55 | 0.25 | 0.01 | 41.55 | 3.10 |
| WC210604 C | 1 | 70.10 | 1.41 | 22.16 | 2.54 | 1.86 | 0.03 | 45.04 | 3.14 |
| | 2 | 55.09 | 1.85 | 50.54 | 1.41 | 1.02 | 0.01 | 100.18 | 0.54 |
| | 3 | 57.45 | 1.49 | 56.59 | 4.32 | 1.11 | 0.02 | 100.22 | 1.06 |
| | 4 | 67.35 | 1.43 | 78.87 | 2.84 | 2.32 | 0.06 | 92.96 | 0.66 |
| | 5 | 54.19 | 5.90 | 53.12 | 0.95 | 1.06 | 0.02 | 116.88 | 2.29 |
| | 6 | 78.49 | 5.74 | 88.27 | 1.26 | 2.01 | 0.02 | 89.32 | 4.45 |
| | 7 | 67.17 | 4.30 | 56.12 | 2.01 | 1.73 | 0.04 | 63.48 | 1.48 |
| MS200405 D | 1 | 100.50 | 2.93 | 32.53 | 1.39 | 0.04 | 0.00 | 131.90 | 7.96 |
| | 2 | 82.19 | 2.20 | 49.54 | 4.29 | 0.04 | 0.00 | 123.44 | 13.92 |
| | 3 | 113.16 | 2.65 | 37.29 | 1.45 | 0.04 | 0.00 | 98.39 | 1.47 |
| | 4 | 73.65 | 0.40 | 39.62 | 5.92 | 0.05 | 0.00 | 84.60 | 6.57 |
| | 5 | 94.40 | 1.24 | 66.19 | 1.63 | 0.04 | 0.00 | 283.69 | 13.80 |
| | 6 | 88.93 | 4.81 | 61.05 | 5.43 | 0.04 | 0.00 | 173.00 | 4.40 |
| ES020407 E | 1 | 133.31 | 14.20 | 24.32 | 3.42 | 2.18 | 0.04 | 36.80 | 9.58 |
| | 2 | 52.42 | 3.70 | 43.10 | 1.07 | 0.62 | 0.01 | 73.01 | 2.30 |
| | 3 | 48.01 | 0.76 | 16.10 | 0.92 | 0.42 | 0.03 | 34.05 | 1.80 |
| | 4 | 156.12 | 31.49 | 31.22 | 2.84 | 2.21 | 0.10 | 41.84 | 6.25 |
| | 5 | 50.68 | 2.85 | 22.30 | 0.00 | 0.57 | 0.02 | 33.17 | 1.15 |
| | 6 | 133.84 | 29.41 | 20.75 | 1.77 | 1.58 | 0.01 | 33.12 | 1.59 |
| GP050407 F | 1 | 114.80 | 6.80 | 39.87 | 1.30 | 0.57 | 0.02 | 149.31 | 1.32 |
| | 2 | 141.62 | 1.27 | 69.77 | 0.64 | 0.76 | 0.03 | 185.26 | 13.29 |
| | 3 | 131.47 | 2.36 | 35.43 | 2.01 | 0.79 | 0.05 | 132.62 | 20.28 |
| | 4 | 120.73 | 10.75 | 73.57 | 12.11 | 1.26 | 0.03 | 187.91 | 3.48 |
| | 5 | 160.91 | 5.72 | 71.52 | 2.55 | 1.13 | 0.07 | 165.01 | 7.11 |
| | 6 | 172.44 | 5.18 | 64.77 | 0.51 | 1.02 | 0.06 | 79.57 | 3.16 |

**Table 5: Carbonyls, Dityrosine, DOPA and TBARS concentrations for plaque sections.**

| Plaque | Section | Carbonyls (nmoles/g plaque) | | Dityrosine (nmoles/g protein) | | DOPA (nmoles/g plaque) | | TBARS (nmoles/g plaque) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| IH120704 A | 1 | 368.85 | 187.28 | 0.85 | 0.17 | 18.29 | 1.33 | 3.69 | 2.75 |
| | 2 | 294.05 | 76.76 | 1.82 | 0.15 | 17.79 | 0.51 | 13.19 | 6.85 |
| | 3 | 245.93 | 38.12 | 1.54 | 0.05 | 14.68 | 0.91 | 8.31 | 10.66 |
| | 4 | 471.43 | 42.10 | 0.26 | 0.02 | 11.63 | 1.45 | 10.04 | 7.35 |
| | 5 | 391.19 | 115.20 | 0.13 | 0.00 | 17.47 | 0.71 | 10.37 | 13.27 |
| | 6 | 327.38 | 41.24 | 0.11 | 0.01 | 13.49 | 1.74 | 7.24 | 16.25 |
| | 7 | 231.43 | 58.76 | 0.00 | 0.00 | 13.13 | 2.67 | 4.82 | 1.67 |
| | 8 | 151.43 | 25.93 | 0.00 | 0.00 | 18.81 | 2.06 | 2.15 | 1.12 |
| HW290304 B | 1 | 163.23 | 26.03 | 1.17 | 0.01 | 56.92 | 11.29 | 0.84 | 0.83 |
| | 2 | 212.01 | 27.47 | 5.11 | 0.25 | 25.96 | 0.29 | 4.77 | 0.75 |
| | 3 | 165.37 | 19.29 | 10.93 | 0.58 | 25.81 | 0.45 | 13.06 | 1.27 |
| | 4 | 76.42 | 16.13 | 42.75 | 1.62 | 32.76 | 1.57 | 12.15 | 2.81 |
| | 5 | 141.56 | 10.53 | 5.08 | 0.28 | 29.44 | 1.95 | 7.05 | 2.03 |
| | 6 | 55.60 | 21.14 | 8.28 | 0.58 | 11.15 | 1.43 | 12.28 | 1.15 |
| | 7 | 171.81 | 31.96 | 7.99 | 0.87 | 16.01 | 4.59 | 4.18 | 0.37 |
| WC210604 C | 1 | 521.00 | 50.27 | 0.00 | 0.00 | 5.58 | 0.66 | 0.44 | 0.44 |
| | 2 | 188.70 | 23.56 | 0.60 | 0.03 | 10.00 | 1.52 | 3.25 | 0.07 |
| | 3 | 221.98 | 50.07 | 1.87 | 0.10 | 4.76 | 0.53 | 10.26 | 0.67 |
| | 4 | 295.19 | 76.91 | 1.18 | 0.15 | 3.27 | 0.93 | 6.68 | 0.85 |
| | 5 | 248.03 | 65.96 | 0.35 | 0.05 | 3.19 | 0.25 | 8.60 | 0.80 |
| | 6 | 304.90 | 50.23 | 1.71 | 0.08 | 5.95 | 0.56 | 2.23 | 0.30 |
| | 7 | 232.80 | 67.45 | 0.66 | 0.05 | 1.48 | 0.16 | 0.22 | 0.07 |
| MS200405 D | 1 | 660.90 | 247.10 | 0.00 | 0.00 | 3.80 | 0.19 | 12.78 | 3.40 |
| | 2 | 299.47 | 228.67 | 10.67 | 0.67 | 35.50 | 13.45 | 6.13 | 1.49 |
| | 3 | 581.77 | 65.39 | 9.70 | 1.02 | 19.30 | 1.69 | 10.87 | 1.44 |
| | 4 | 468.92 | 56.27 | 15.66 | 0.48 | 22.99 | 1.47 | 6.27 | 1.13 |
| | 5 | 347.48 | 81.05 | 16.02 | 0.37 | 2.22 | 1.39 | 6.66 | 1.06 |
| | 6 | 311.72 | 135.44 | 1.14 | 0.05 | 9.21 | 4.56 | 0.08 | 0.08 |
| ES020407 E | 1 | 338.52 | 193.02 | 0.00 | 0.00 | 13.92 | 9.17 | 24.94 | 1.53 |
| | 2 | 200.71 | 64.96 | 0.00 | 0.00 | 3.20 | 2.25 | 10.80 | 1.52 |
| | 3 | 56.36 | 41.51 | 0.00 | 0.00 | 4.22 | 0.58 | 13.54 | 2.49 |
| | 4 | 453.51 | 93.19 | 0.00 | 0.00 | 3.93 | 2.21 | 10.20 | 2.45 |
| | 5 | 255.05 | 109.38 | 0.00 | 0.00 | 6.32 | 1.75 | 17.82 | 3.73 |
| | 6 | 352.94 | 300.23 | 0.00 | 0.00 | 13.96 | 7.30 | 8.35 | 1.41 |
| GP050407 F | 1 | 300.54 | 9.82 | 6.08 | 0.41 | 6.20 | 0.88 | 11.63 | 1.72 |
| | 2 | 401.12 | 76.91 | 9.59 | 0.27 | 12.35 | 2.76 | 13.55 | 2.54 |
| | 3 | 334.54 | 66.55 | 9.96 | 2.03 | 8.97 | 1.11 | 6.03 | 0.74 |
| | 4 | 277.78 | 35.77 | 11.75 | 4.29 | 10.76 | 0.87 | 23.65 | 3.50 |
| | 5 | 693.88 | 40.82 | 10.33 | 2.21 | 12.19 | 0.86 | 22.22 | 0.47 |
| | 6 | 331.54 | 131.63 | 7.44 | 1.51 | 9.99 | 0.88 | 15.87 | 0.38 |

# Appendix II

The following papers are entitled “Dissociation of neopterin and 7,8-dihydroneopterin from plasma components before HPLC analysis” and “Potential to inhibit growth of atherosclerotic plaque development through modulation of macrophage neopterin/7,8-dihydroneopterin synthesis”. The first was generated entirely from this Masters thesis; the second includes one figure (Figure 2) that was generated from data produced as a part of this Masters thesis.

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Short communication

# Dissociation of neopterin and 7,8-dihydroneopterin from plasma components before HPLC analysis

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**Abstract**

Measurement of plasma neopterin by HPLC with fluorescence detection is used clinically as a marker of immune cell activation in the management of a number of disease pathologies. HPLC analysis of neopterin requires the acidic removal of plasma proteins but we have found that 7,8-dihydroneopterin is oxidised to neopterin with varying yield. Using acetonitrile as the precipitant, we have measured substantially higher quantities of both total neopterin (7,8-dihydroneopterin and neopterin) and neopterin from plasma of healthy and septicemia patient's. Total neopterin concentrations were on average 50% and 200% greater in healthy and septicemia subjects, respectively, when measured after acetonitrile precipitation compared to trichloroacetic acid. Our data suggests that some pterin co-precipitates with proteins during acid treatment.


*Keywords:* Neopterin; 7,8-Dihydroneopterin; Protein precipitation; HPLC; Acetonitrile; Trichloroacetic acid

## 1. Introduction

Neopterin and its reduced form 7,8-dihydroneopterin, are synthesised and released primarily by human macrophages when stimulated with γ-interferon [1]. Elevated plasma and urinary neopterin levels are used as a marker of inflammation and the immune response. Measurement of plasma and urine neopterin are reportedly used in the clinical management of HIV infection [2], autoimmune diseases [3], bacterial infections [4] and post-operative transplant patients [5] to name a few of many studies. 7,8-Dihydroneopterin has been shown by us and other laboratories to be a potent antioxidant in vitro capable of inhibiting oxidative damage to both cells, proteins and lipoproteins [6–12].

The level of neopterin in biological fluids is measured either by ELISA [13] or by HPLC with fluorescence detection of the highly fluorescent neopterin. Though the initial purchase price of a HPLC is relatively high compared to ELISA equipment, the cost in consumables for each individual sample is relatively inexpensive making it the method of choice in many research laboratories.

HPLC analysis of plasma or tissue requires the removal of proteins. Original methods used ion exchange solid phase extraction to collect and concentrate the neopterin [14,15], but with changes to solid phase manufacturing these methods appear to have become unreliable and technically demanding. The more common methods now employed remove sample proteins by acid precipitation prior to HPLC analysis [16]. However, under acidic conditions, 7,8-dihydroneopterin is oxidised to neopterin with varying yield [14]. This oxidation can be partially prevented by the addition of ascorbate prior to acid precipitation but is not completely effective. Total neopterin levels (7,8-dihydroneopterin + neopterin) are usually determined by oxidising the 7,8-dihydroneopterin to highly fluorescent neopterin with an acidic iodide solution. We here report that the acid precipitation step also removes or co-precipitates varying levels of pterin with the plasma proteins which results in an under-estimation of neopterin and total neopterin levels in plasma. The use of the solvent acetonitrile to precipitate plasma proteins appears to eliminate these problems.

*Abbreviation:* TCA, trichloroacetic acid.

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## 2. Experimental

### 2.1. Chemical and reagents

Chemicals and reagents were AR grade or better and, unless otherwise stated, were obtained from either Sigma Chemical Company (USA) or BDH Chemicals New Zealand Ltd. Neopterin and 7,8-dihydroneopterin was supplied by Schirck's Laboratories (Switzerland). All solutions were prepared with high purity water from a NANOpure ultrapure water system, supplied by Barnstead/Thermolyne (IA, USA).

### 2.2. Sample collection

Blood samples were obtained by venipuncture from 10 randomly selected septicemia patients and 5 healthy control subjects. Septicemia patients were undergoing aminoglycoside therapy and blood samples were collected as part of their routine monitoring. All blood samples were surplus to clinical requirements and were anonymised before release for neopterin analysis as part of the diagnostic laboratory ethics approval for assay validation or establishing a reference range. This study has been approved by the Upper South A Regional Ethics Committee, New Zealand. Plasma was shielded from light and processed under red light illumination to prevent oxidative loss by UV light. The plasma was prepared by centrifugation and stored at −80 °C until analysis.

### 2.3. Precolumn plasma preparation

Where possible all treatments were carried out under red light illumination to prevent oxidative loss by UV light. Two differing sample preparation methods were employed for neopterin measurement. The first involved acid precipitation of protein by adding 100 μL of plasma, 10 μL of 0.6 M ascorbate and 10 μL of 50% trichloroacetic acid (TCA) followed by vortexing then centrifugation (4 °C and 10,300 × *g* for 15 min); 100 μL of the acid supernatant was then placed in an autosampler vial for HPLC analysis. The second method employed acetonitrile to precipitate the protein. 100 μL of plasma was combined with 100 μL of 100% acetonitrile, vortexed and centrifuged (4 °C and 10,300 × *g* for 10 min). 100 μL of the supernatant was then transferred to an autosampler vial for HPLC analysis.

For total neopterin analysis an oxidation step was included to convert 7,8-dihydroneopterin to neopterin for detection following the protein precipitation and centrifugation steps. 10 μL of acidic iodide solution (5.4% $I_2$/10.8% KI in 1 M HCl) was added to the supernatant and incubated for 20 min at room temperature in the dark. 10 μL of 0.6 mM ascorbate was added to oxidise the iodine before centrifugation of the samples (4 °C and 10,300 × *g* for 5 min). With the TCA treatment method, the ascorbate was not added before protein precipitation during total neopterin analysis. Except during the 7,8-dihydroneopterin oxidation to neopterin, all sample solutions were kept between 0 °C and 4 °C.

### 2.4. HPLC analysis

HPLC measurement of neopterin was performed using a Shimadzu Sil-10A HPLC with autosampler and RF-10Axls fluorescence detector [17]. 10 μL of sample was injected onto a Phenomenex Develosil reverse phase ODS-MG-5 4.2 mm × 250 mm column with a mobile phase of 5% methanol in 20 mM ammonium phosphate pH 6.0 pumped at 1 mL/min. Neopterin was detected by its native fluorescence at 438 nm, excitation 353 nm. The concentration and identity of the eluted neopterin was confirmed by comparison to a commercial standard and quantified by peak area using Shimadzu Class VP software. All analysis was conducted in triplicate and data is displayed as the mean ± the standard error of the mean of triplicate treatments.

## 3. Results and discussion

The use of acetonitrile to remove plasma proteins markedly improved the resolution and signal to noise ratio seen during HPLC compared to TCA treatment (Fig. 1). TCA-treated samples also showed a number of additional contaminant peaks during chromatography. With the acetonitrile-treated samples, neopterin was consistently observed to elute 1 min sooner than the neopterin from TCA-treated samples due to the presence of 50% acetonitrile in the injected sample. The neopterin peak identity was confirmed by spiking plasma samples with authentic neopterin. The recovery of this added neopterin was 100% showing that the changed elution time was not effecting the peak height.

In the acetonitrile-treated sample's chromatogram (Fig. 1B), the neopterin peak area is smaller than that seen in the chromatogram of the TCA-treated sample (Fig. 1A). This is due to the dilution of sample which occurs with the acetonitrile treatment. When dilution factors are taken into account the apparent concentration of neopterin in the plasma from the TCA-treated sample is 31.4 nM and in the acetonitrile-treated sample 55.3 nM. This apparent increase in neopterin levels with acetonitrile treatment was generally observed with all plasma samples examined. This increase in neopterin with acetonitrile treatment was not observed using protein free buffers suggesting the effect was due to the actual precipitation of the protein.

The linearity of the acetonitrile treatment and analysis was confirmed by measuring different concentrations of neopterin between 5 nM and 2 μM. The calibration curve ($y = 1.019x - 0.015$) was linear over this range with a correlation coefficient of $r^2 = 1.00$. Within-run precision, evaluated by 10 consecutive injects from the same plasma sample, and between-run precision, evaluated by injects on 6 separate days from the same plasma pool, showed repeatability of the assay is good (within-run CV% < 2.9; between-run CV% < 3.8).

Acidic conditions have been previously reported to cause significant oxidation of 7,8-dihydroneopterin to neopterin [14]. With TCA we observed up to 60% of 7,8-dihydroneopterin being oxidised to neopterin (data not shown). Similar problems were also encountered using perchloric acid. The effect of this oxidation would not be apparent when dealing with

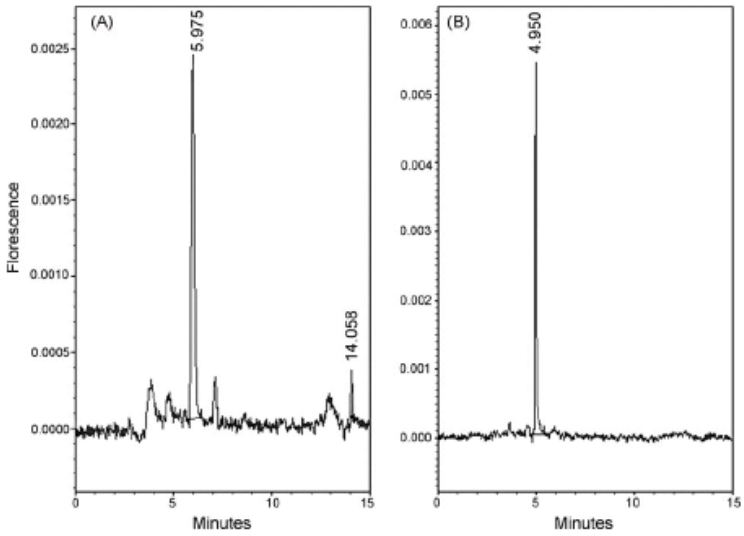


Fig. 1. HPLC chromatograms of plasma neopterin (without iodine oxidation) from (A) TCA- and (B) acetonitrile-treated plasma sample from a selected septicemia patient.

neopterin samples alone, but would cause a substantial underestimate of 7,8-dihydroneopterin concentrations and a slight additional overestimate of neopterin when working with biological samples. Ascorbate was found to reduce this oxidation but was not completely effective. This effect of ascorbate was previously described by Werner et al. [14]. Therefore acetonitrile was examined as an alternative to acid precipitation of proteins. We found that 7,8-dihydroneopterin was not oxidised by treatment with acetonitrile making it a more suitable reagent for protein precipitation (data not shown).

Analysis of 10 septicemia patients and 5 apparently healthy controls showed the acetonitrile treatment consistently returned higher levels of neopterin and total neopterin than the acid based treatment (Figs. 2 and 3). The acetonitrile precipitation treatment gave on average a 20 nM increase in neopterin levels compared to that obtained using TCA for both the healthy controls and septicemia patients (Figs. 2A and 3A). However, with total neopterin analysis, where the 7,8-dihydroneopterin is oxidised to neopterin, the increase in neopterin due to the acetonitrile treatment, compared to TCA, was less consistent (Fig. 3B). With healthy controls acetonitrile treatment gave on average a 50% increase in total neopterin compared to TCA, but this increase ranged from 4.3 nM to 12.9 nM.

With septicemia patients the measured total neopterin level also increased with acetonitrile treatment (Fig. 2B) but by approximately 100% compared to the TCA-treated samples. The size of this increase ranged from 15 nM to 120 nM.

TCA treatment shows a picture of healthy subjects having the majority of the pterin as 7,8-dihydroneopterin (75%) (Fig. 4) which is close to the published 2:1 ratio [18]. In the septicemia patients, this ratio is reversed where the majority of the pterin is neopterin (83%). This does agree with the hypothesis that

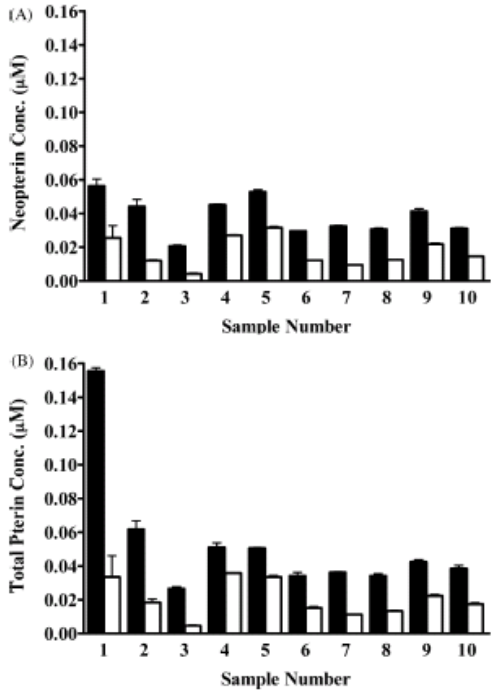


Fig. 2. Plasma neopterin (A) and total neopterin (B) concentrations for 10 septicemia patients. Samples were prepared for analysis using either acetonitrile (■) or TCA (□) for protein precipitation before HPLC analysis as described in Section 2. Values graphed are the mean + S.E. of three replicates for each sample.

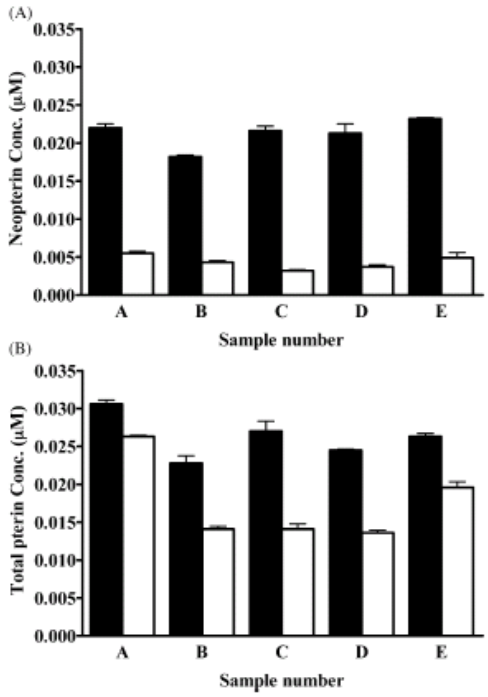


Fig. 3. Plasma neopterin (A) and total neopterin (B) concentrations for five apparently healthy control subjects. Samples were prepared for analysis using either acetonitrile (■) or TCA (□) for protein precipitation before HPLC analysis. Values graphed are the mean + S.E. of three replicates for each sample.

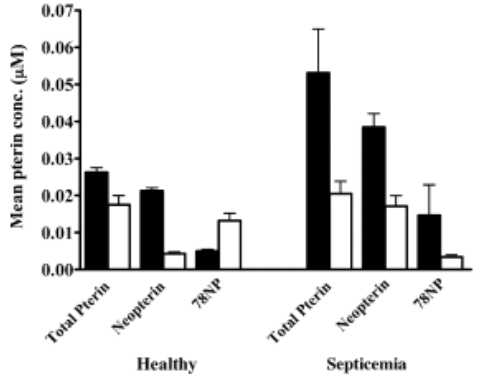


Fig. 4. Mean plasma total neopterin, neopterin and 7,8-dihydroneopterin concentrations for the two experimental groups (septicemia and healthy controls). The 7,8-dihydroneopterin concentration for each sample was calculated by subtracting the total neopterin concentration from the plasma neopterin concentration. The figure shows the mean of the acetonitrile-treated (■) and TCA-treated (□) samples from Figs. 2 and 3.

7,8-dihydroneopterin can be oxidised to neopterin by oxidants produced during inflammation. However, the TCA data does not show a significant overall increase in total neopterin when comparing the healthy controls and the septicemia patients (Fig. 4). This is not consistent with what is known about pterin release from macrophages during the immune response [1,19]. The acetonitrile data shows the overall level of pterins in the plasma increasing 203% with the level of 7,8-dihydroneopterin increasing from 19% to 28% which would be consistent with increased production by macrophage cells, especially if one of the roles of 7,8-dihydroneopterin is to provide protection to cells and biomolecules [9,20,21]. The overall increase in neopterin/7,8-dihydroneopterin is also consistent with the hypothesis that neopterin increases the potency of various cytotoxic agents [22–24].

The variation in levels of neopterin and total neopterin between the acetonitrile-treated and TCA-treated plasma samples cannot be fully explained by the acid-induced oxidation of 7,8-dihydroneopterin to neopterin, as the levels of both increased dramatically with the acetonitrile treatment. We suggest that in addition to the acid oxidation, a varying level of pterin is co-precipitating with the serum proteins and is lost from the sample.

Our data suggests that the current threshold of 10 nM, above which inflammation is suspected, should be raised when using acetonitrile for protein removal. The level at which this threshold is set will require further analysis of a larger pool of healthy subjects than presented here.

## 4. Conclusion

The use of acetonitrile rather than acidic conditions to precipitate and remove plasma proteins provides a significant increase in the amount of total neopterin and neopterin measured in clinical samples. The procedure also allows the accurate calculation of plasma 7,8-dihydroneopterin levels as this compound is not oxidised to neopterin during acetonitrile treatment. The chromatography of the acetonitrile-treated samples is also significantly improved without contamination from TCA.

## Acknowledgement

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REVIEW

# Potential to inhibit growth of atherosclerotic plaque development through modulation of macrophage neopterin/7,8-dihydroneopterin synthesis

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The rise in plasma neopterin observed with increasing severity of vascular disease is a strong indicator of the inflammatory nature of atherosclerosis. Plasma neopterin originates as the oxidation product of 7,8-dihydroneopterin secreted by γ-interferon stimulated macrophages within atherosclerotic plaques. Neopterin is increasingly being used as a marker of inflammation during clinical management of patients with a range of disorders including atherosclerosis. Yet the role of 7,8-dihydroneopterin/neopterin synthesis during the inflammatory process and plaque formation remains poorly understood and controversial. This is partially due to the unresolved role oxidants play in atherosclerosis and the opposing roles of 7,8-dihydroneopterin/neopterin. Neopterin can act as pro-oxidant, enhancing oxidant damage and triggering apoptosis in a number of different cell types. Neopterin appears to have some cellular signalling properties as well as being able to chelate and enhance the reactivity of transition metal ions during Fenton reactions. In contrast, 7,8-dihydroneopterin is also a radical scavenger, reacting with and neutralizing a range of reactive oxygen species including hypochlorite, nitric oxide and peroxyl radicals, thus protecting lipoproteins and various cell types including macrophages. This has led to the suggestion that 7,8-dihydroneopterin is synthesized to protect macrophages from the oxidants released during inflammation. The oxidant/antioxidant activity observed *in vitro* appears to be determined both by the relative concentration of these compounds and the specific chemistry of the *in vitro* system under study. How these activities might influence or modulate the development of atherosclerotic plaque *in vivo* will be explored in this review.
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Keywords: atherosclerosis; inflammation; neopterin; 7,8-dihydroneopterin; low-density lipoprotein; antioxidant; pro-oxidant; macrophage-apoptosis; interferon; indoleamine-2,3-dioxygenase

Abbreviations: AAPH, 2,2′-azobis(amidinopropane) dihydrochloride; GM-CSF, colony-stimulation factor; GTP, guanosine 5′-triphosphate; 3HAA, 3-hydroxyanthranilic acid; HMDM, human monocyte-derived macrophages; HPLC, high-performance liquid chromatography; IDO, indoleamine 2,3-dioxygenase; iNOS, inducible nitric oxide synthase; LDL, low-density lipoprotein; oxLDL, oxidized low-density lipoprotein; TNF-α, tumour necrosis factor-α

## Introduction

Atherosclerotic plaques are sites of chronic inflammation (Libby *et al.*, 2002). This is clearly shown by the presence of large numbers of immune cells including macrophages, and various inflammatory markers within the plaque and plasma of patients. Although much attention has been given to the elevation of C-reactive protein in the plasma of heart disease patients, the inflammation marker neopterin is also significantly elevated in patients with vascular disease (Tatzber *et al.*, 1991; Schumacher *et al.*, 1992; Rudzite *et al.*, 2005). Neopterin is synthesized and released from γ-interferon activated macrophages as part of the inflammation process. Neopterin has been investigated as a marker of immune cell activation in a wide range of diseases as it is relatively easy to analyse by high-performance liquid chromatography (HPLC) and is generated by one of the key inflammatory cells, the macrophage, during inflammation.

The macrophage is considered to be the key cell in the development and growth of atherosclerotic plaques (Carpenter *et al.*, 1995; Steinberg, 1995). Both fatty streaks and advance plaques are rich in macrophages. Macrophage cells release a range of proteolytic and oxidizing agents including superoxide, hydrogen peroxide, lipid peroxides, lipoxygenases and possibly hypochlorite (Ylaherttuala *et al.*, 1989; Schewe and Kuhn, 1991; Chisolm *et al.*, 1999). All these agents have been shown *in vitro* to alter the low-density lipoprotein (LDL) found within the tissue bed of the intima (innermost layer of the artery wall) to oxidized LDL (oxLDL).

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OxLDL is readily taken up by macrophages via scavenger receptors in a relatively non-regulated process. The uptake of oxLDL causes the macrophages to differentiate into cholesterol-loaded macrophages often referred to as 'foam cells' due to their foamy appearance (Goldstein *et al.*, 1979; Hoff *et al.*, 1989; Steinbrecher *et al.*, 1989). A number of the oxidized fatty acids within oxLDL are chemotactic to monocytes while the oxysterols and fatty acid peroxides are toxic to macrophages, fibroblasts, smooth muscle cells and endothelium, all of which make up advanced plaques (Lassila, 1993; Hegyi *et al.*, 1996). The death and lysis of all these cells, especially lipid-loaded macrophages, results in the formation of a lipid-rich necrotic core region within atherosclerotic plaque. Atherosclerotic plaques containing lipid-rich necrotic cores are prone to rupture and thrombus formation (Van Der Wal and Becker, 1999). The finding that plasma neopterin levels increase with atherosclerotic plaque formation shows a direct link between macrophage activation and plaque development.

This oxidative model of heart disease has been heavily criticized in recent years due to the failure of various antioxidant intervention trials to demonstrate a significant level of protection. This criticism ignores the fact that ascorbate and tocopherol levels are tightly controlled *in vivo* as both are potent pro-oxidants in elevated concentrations (Bowry and Stocker, 1993; Clement *et al.*, 2001). Therefore, neither are good therapeutic agents from a pharmacological point of view. The criticism also ignores the fact that oxidized lipids and proteins are both found within plaques and these agents have reasonably well-defined inflammatory effects on cells (Carpenter *et al.*, 1995; Woods *et al.*, 2003). LDL oxidation may not initiate plaque formation, but its presence will significantly and detrimentally affect the functioning of cells within the artery wall. The rise of C-reactive protein and neopterin clearly show atherosclerosis has a significant inflammatory component. It is therefore possible that agents which alter this inflammatory process will slow or prevent plaque growth, or the formation of more complex plaques. *In vitro* both neopterin and its reduced form 7,8-dihydroneopterin have been shown to have significant effects on oxLDL formation and cell death. Unlike ascorbate, tocopherol or dietary flavonoids, neopterin and 7,8-dihydroneopterin are generated within the plaque and therefore do not need to be delivered to the site of artherogenesis.

## Neopterin and 7,8-dihydroneopterin synthesis

Neopterin is the oxidized product of 7,8-dihydroneopterin, a pterin synthesized by primate macrophages when stimulated with γ-interferon (Muller *et al.*, 1991; Wachter *et al.*, 1992). GTP-cyclohydrolase is one of the many enzymes upregulated by γ-interferon. The enzyme catalyses the breakdown of GTP to 7,8-dihydroneopterin triphosphate. In primate macrophages, low levels of the enzyme 6-pyruvoyltetrahydropterin synthetase causes a build up of 7,8-dihydroneopterin triphosphate which is then released as 7,8-dihydroneopterin due to the action of intracellular phosphatases (Schoedon *et al.*, 1987). 7,8-Dihydroneopterin diffuses out of the activated

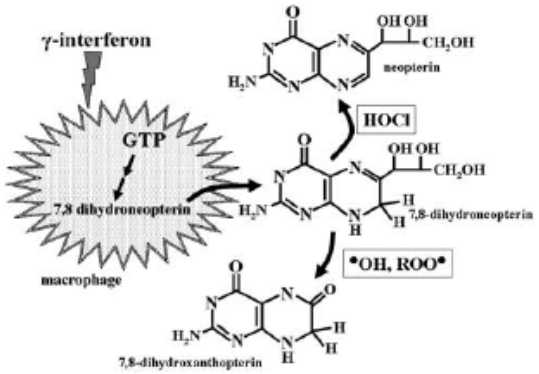


**Figure 1** γ-Interferon stimulation of macrophages causes the enzymatic breakdown of intracellular GTP to dihydroneopterin which can diffuse from the cell and either be oxidized to the high fluorescent neopterin by HOCl or to 7,8-dihydroxanthopterin by reactive oxygen species.

macrophages into the intracellular spaces and finally the plasma (Figure 1). Some of the 7,8-dihydroneopterin is oxidized to the highly fluorescent neopterin. The release of 7,8-dihydroneopterin and neopterin is specific to monocytes, macrophages and dendritic cells (Wirleitner *et al.*, 2002) although kidney epithelial cells have also been observed to release neopterin (Moutabarrik *et al.*, 1994).

Primate macrophages (including human) are unique in this response, as non-primate macrophages convert 7,8-dihydroneopterin-triphosphate into inducible nitric oxide synthase (iNOS) cofactor 5,6,7,8-tetrahydrobiopterin. As a result primate macrophages, unlike mouse macrophages, do not generate significant levels of nitric oxide when stimulated with interferon but release the highly fluorescent neopterin.

Interestingly, the main reaction generating neopterin from 7,8-dihydroneopterin is oxidation by hypohalous acids such as HOCl (Widner *et al.*, 2000). Proton abstraction from carbon-7 and nitrogen-8 of 7,8-dihydroneopterin generates neopterin. Neutrophils and possibly macrophages release significant amounts of HOCl during inflammation (Schraufstatter *et al.*, 1990) suggesting much of the neopterin measured in plasma has come from sites of inflammation where HOCl is being released. The presence of neopterin within plasma is further indication of the inflammatory origin of these pterins.

## Clinical measurement

The central role of γ-interferon communicating between T cells and macrophages with the subsequent release of neopterin make plasma neopterin measurements an ideal method for gauging immune activation within a patient (Wachter *et al.*, 1989, 1992). The injection of γ-interferon causes a rapid and sustained rise in plasma neopterin levels (Muller *et al.*, 1991). Neopterin is easily measured in plasma and urine by HPLC due to its extremely high fluorescence

(Werner *et al.*, 1987; Rippin, 1992) although many clinical laboratories also use immuno-based methods such as enzyme-linked immunosorbent assay to measure neopterin (Westermann *et al.*, 2000).

In response to infection, plasma neopterin levels rise rapidly in parallel with C-reactive protein levels, well before a patient becomes sero-positive. Measurements of plasma neopterin levels are used as a clinical tool to assess efficacy of treatments for a range of infections including malaria (Reibnegger *et al.*, 1984; Awandare *et al.*, 2006), tuberculosis (Fuchs *et al.*, 1984; Yuksekol *et al.*, 2003) and human immunodeficiency virus (Fuchs *et al.*, 1988) to name a few. As elevated neopterin levels appear to occur with most inflammatory conditions, some hospitals measure plasma neopterin as a primary screen for blood donations (Strohmaier *et al.*, 1996). The monitoring of plasma neopterin has also been used in the study and management of cancer (Reibnegger *et al.*, 1991), autoimmune disease (Reibnegger *et al.*, 1986; Schroecksnadel *et al.*, 2003) and transplant patients (Margreiter *et al.*, 1983; Yokoyama *et al.*, 2002) where the rise in plasma or urine neopterin levels can give clinicians adequate warning of allograft rejection enabling them to alter immunosuppressant treatment.

Although plasma neopterin is not generally used in the management of vascular disease, there is a growing amount of knowledge on its value. Serum neopterin is elevated in patients with unstable angina and acute myocardial infarction (Tatzber *et al.*, 1991; Schumacher *et al.*, 1992, 1997). There is also a strong correlation between serum neopterin and the thrombolysis in myocardial infarction risk score in patients with unstable angina, or acute myocardial infarction (Johnston *et al.*, 2006). The anti-inflammatory affects of HMG-CoA inhibiting statin drugs is also demonstrated by the lowering of serum neopterin levels (Neurauter *et al.*, 2003; Walter *et al.*, 2003).

Surprisingly, although the Web of Science lists over 2000 references relating to neopterin, the exact role of neopterin synthesis and release by monocyte-derived cells is not understood. It has been suggested that neopterin and 7,8-dihydroneopterin are synthesized as pro-oxidants, enhancing oxidant production and cell death in combination with tumour necrosis factor (TNF). In contrast, 7,8-dihydroneopterin has also been reported to act as an antioxidant, protecting biomolecules and macrophages from oxidants released during inflammation. Neopterin release has also been suggested to provide a feedback to T cells on the level of immune activation occurring. There is good experimental evidence supporting all these mechanisms and all have the potential to alter atherosclerotic plaque development.

## Inhibition of LDL oxidation

Like many reducing agents, 7,8-dihydroneopterin rapidly reacts with free radical and oxidizing species. This was first noted with chemiluminescence-based assays where reduced pterins, including 7,8-dihydroneopterin, were found to inhibit the luminescence signal from superoxide and hydrogen peroxide (Shen, 1994). When 7,8-dihydroneopterin was sent to Esterbauer's laboratory in Graz, Austria, it was soon found that 7,8-dihydroneopterin was a potent inhibitor of metal ion and aqueous peroxyl radical (2,2′-azobis(amidinopropane) dihydrochloride (AAPH))-mediated LDL oxidation (Gieseg *et al.*, 1995). A few years earlier, tetrahydroneopterin had been shown to inhibit xanthine/xanthine oxidase and phorbol myristate acetate stimulated macrophage superoxide production (Kojima *et al.*, 1992) and inhibiting linoleic acid oxidation, so in hindsight it was not a surprising finding. What was unexpected was that 7,8-dihydroneopterin could out-compete the LDL tocopherol (vitamin E) for the primary propagating lipid radical. 7,8-Dihydroneopterin had a high reaction rate with peroxyl radicals, but this did not explain how a water-soluble compound could react with the lipid radicals within the LDL particle (Oettl *et al.*, 1997). This mechanism remains unresolved but it has been suggested that 7,8-dihydroneopterin may bind or become compartmentalized on the LDL (Gieseg *et al.*, 1995, 2003). Studies on the inhibition of peroxynitrite oxidation of LDL by 7,8-dihydroneopterin suggested that 7,8-dihydroneopterin might diffuse into the phospholipid layer of the LDL particle (Herpfer *et al.*, 2002). With both peroxynitrite and copper-mediated LDL oxidation, the protective effect of 7,8-dihydroneopterin was enhanced by preincubation before addition of the oxidant. Nitric oxide and peroxynitrite have been implicated in modification of LDL within plaques, especially the nitration of some amino-acid side chains like tyrosine which is effectively blocked by 7,8-dihydroneopterin (Widner *et al.*, 1998; Oettl *et al.*, 2004). Protein hydroperoxides and their decay product carbonyls make a large and significant contribution to the oxidative damage occurring on the LDL particle (Yan *et al.*, 1997; Gieseg *et al.*, 2003). This protein oxidation on the ApoB100 moiety of the LDL particle is effectively inhibited by 7,8-dihydroneopterin through scavenging of lipid peroxyl radicals.

Although oxidative levels of transition metals and peroxyl radicals appear to exist within atherosclerotic plaques, copper ion and AAPH-peroxyl radical-mediated LDL oxidation appears relatively artificial when carried out in dilute buffers. There is evidence that ceruloplasmin-bound copper ions alone maybe able to oxidize LDL within atherosclerotic plaques (Shukla *et al.*, 2006). Macrophages and other cells are considered by many to be the key mediators of oxLDL formation within atherosclerotic plaques. *In vitro* cell-mediated oxLDL formation is either superoxide dependent or independent depending on the cell type and condition used (Jessup *et al.*, 1993; Aviram *et al.*, 1996). With monocyte-like THP-1 cells and human monocyte-derived macrophages (HMDM), oxLDL formation is totally inhibited by micromolar concentrations of 7,8-dihydroneopterin (Gieseg *et al.*, 2003; Gieseg and Cato, 2003). As THP-1 cell-mediated oxidation is independent of superoxide formation, it is likely inhibition is due to scavenging the lipid peroxyl radicals in the LDL.

However, 7,8-dihydroneopterin can also accelerate LDL oxidation if added after initiation of the oxidation process due to its role as a reducing agent (Herpfer *et al.*, 2002; Greilberger *et al.*, 2004). 7,8-Dihydroneopterin reduces oxidized metal ions, which increases the pool of reduced copper ions available to react with polyunsaturated lipids and peroxyl radicals within the LDL. The same effect was

reported in phosphate-buffered solution containing low levels of iron where 7,8-dihydroneopterin enhanced the rate of hydroxyl radical generation (Oettl *et al.*, 1999). So like tocopherol, under the right conditions, 7,8-dihydroneopterin may actually accelerate oxLDL formation within plaque (Bowry *et al.*, 1992; Niu *et al.*, 1999).

It is interesting to note that 7,8-dihydroneopterin is not the only compound generated by $\gamma$-interferon-stimulated macrophages, which can inhibit LDL oxidation. The enzyme indoleamine 2,3-dioxygenase (IDO) is also upregulated by interferon stimulation. IDO catalyses the degradation of the amino-acid tryptophan to a range of products including kynurenine and 3-hydroxyanthranilic acid (3HAA) (Werner-Felmayer *et al.*, 1990). Like 7,8-dihydroneopterin, 3HAA inhibits copper and AAPH-peroxyl radical-mediated LDL oxidation at micromolar concentrations. At high tryptophan concentrations, interferon-stimulated macrophages generated enough 3HAA to inhibit macrophage-mediated LDL oxidation (Christen *et al.*, 1994; Thomas *et al.*, 1996).

*In vitro*, interferon stimulation of macrophages fails to generate enough 7,8-dihydroneopterin to inhibit the LDL oxidation. Tissue culture levels are in the low nanomolar range although can be elevated to over 100 nM with the addition of either colony-stimulation factor (GM-CSF), phorbal esters, or IL-6 to the interferon-containing media. Yet our studies have shown the level of neopterin within atherosclerotic plaques is in the micromolar range (Figure 2). This suggests that additional factors are involved within plaques to generate these elevated levels of neopterin. The fact that the plaques appear to be the source of serum neopterin also suggests a high level of 7,8-dihydroneopterin synthesis within the plaque.

On this basis 7,8-dihydroneopterin and 3HAA, generated by the interferon-stimulated macrophages should inhibit plaque formation and LDL oxidation. Yet oxidized lipids and proteins can be detected within atherosclerotic plaques and high levels of neopterin. This suggests there is some sort of balance between oxidant and antioxidant, which becomes disturbed in regions of the artery wall. The correction of this balance through the control of macrophage antioxidant/oxidant generation may slow or inhibit plaque growth.

## Protection from cellular toxicity

Sites of inflammation, including atherosclerotic plaques, contain a range of reactive oxidants which can trigger apoptotic and necrotic death within cells (Martinet and Kockx, 2001). OxLDL is particularly cytotoxic to a range of cells including macrophages (Clare *et al.*, 1995; Marchant *et al.*, 1995). The finding that reduced pterins such as 7,8-dihydroneopterin can protect LDL and other biomolecules leads to the hypothesis that 7,8-dihydroneopterin was synthesized by interferon-stimulated macrophages to protect these antigen-presenting cells from the oxidants encountered within an inflammatory site (Schroder *et al.*, 1987; Kojima *et al.*, 1992; Gieseg *et al.*, 1995, 2002). The fact that 3HAA is also generated by macrophages during inflammation further supports this hypothesis (Christen *et al.*, 1990; Werner-Felmayer *et al.*, 1990). Micromolar concentrations of

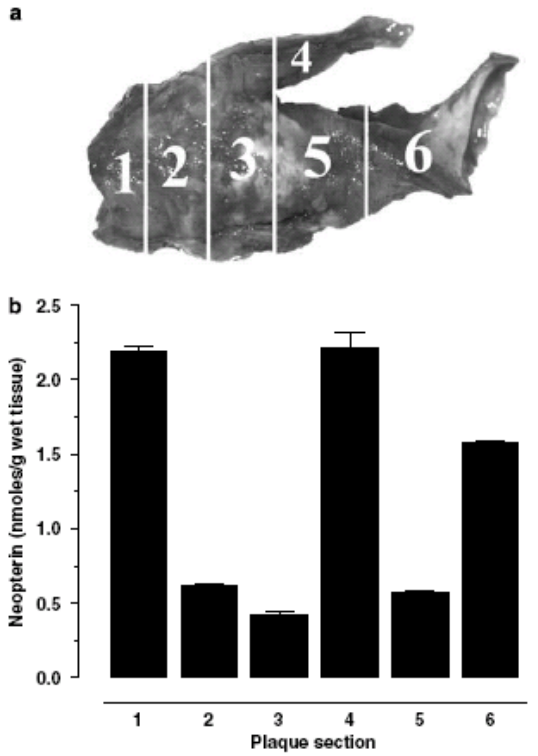


**Figure 2** Concentration of neopterin across the length of a carotid atherosclerotic plaque. The plaque was removed via carotid endarterectomy from the left common carotid artery. The plaque was sectioned longitudinally into six slices, 2–3 mm thick, from the proximal to the distal end (a). Sections 1–2 represent the pre-bifurcation region, sections 3 and 5 the bifurcation, section 4 the secondary branch and section 6 the post-bifurcation of the primary branch. The sections were homogenized and neopterin levels measured using HPLC (high-performance liquid chromatography) with fluorescence detection (b). The plaque sample was highly calcified and caused a 90% stenosis in the artery.

7,8-dihydroneopterin do inhibit cellular damage to red blood cells and the monocyte like human-derived U937 cells, from a range of oxidants including hydrogen peroxide, hypochlorite, aqueous peroxyl and direct plasma membrane oxidation by ferrous ions (Gieseg *et al.*, 2000, 2001a, b). There is evidence that all these oxidants occur within sites of inflammation and atherosclerotic plaques (Brown *et al.*, 1997; Leeuwenburgh *et al.*, 1997; Hazen *et al.*, 2000; Stadler *et al.*, 2004).

OxLDL-induced necrosis in monocyte like U937 cells is inhibited by micromolar concentrations of 7,8-dihydroneopterin (Baird *et al.*, 2005). The mechanism appears to involve the protection of the intracellular glutathione pool thus maintaining the redox status of the cell. The same mechanism also appears to occur with 7,8-dihydroneopterin protection of HMDM cells where necrosis is triggered by the loss of glutathione by oxLDL exposure (unpublished data).

Surprisingly, 7,8-dihydroneopterin does not protect THP-1 cells, another human-derived monocyte-like cell line, from

oxLDL or AAPH-peroxyl radical-induced cell death (Baird *et al.*, 2005). Although 7,8-dihydroneopterin protects HMDM cells from oxLDL, it provides little or no protection against AAPH-peroxyl radicals (Firth *et al.*, 2007). Serum albumin oxidation by AAPH-peroxyl radicals is completely inhibited by 7,8-dihydroneopterin through scavenging of aqueous peroxyl radicals (Platt and Gieseg, 2003), yet with THP-1 cells and HMDM cells this inhibition did not appear to occur. This suggests that 7,8-dihydroneopterin protects cells through either a specific intracellular radical scavenger mechanism, or by acting as a form of signal molecule, which triggers various anti-necrotic processes within the cell.

Although neopterin is described as the oxidation product of 7,8-dihydroneopterin, the scavenging of hydrogen peroxide, superoxide and peroxyl radicals generates 7,8-dihydroxanthopterin (Gieseg *et al.*, 2002). This compound forms through the loss of the trihydroxypropyl side chain attached to carbon-6 of 7,8-dihydroneopterin (Figure 1). The reaction appears to occur through a retro–aldol reaction initiated by the abstraction of an atom of hydrogen from the middle carbon hydroxyl group on the 7,8-dihydroneopterin side chain. Unfortunately, 7,8-dihydroxanthopterin is not fluorescent and is difficult to detect in plasma. Currently, the only mechanism to describe the generation of neopterin from 7,8-dihydroneopterin *in vivo* is the reaction with hypochlorite. Hypochlorite is released from activated neutrophils and possibly macrophages during inflammation (Chisolm *et al.*, 1999). This highly reactive oxidant has been implicated in the modification of LDL within plaques and killing cells (Daugherty *et al.*, 1994; Fabjan *et al.*, 2001; Whiteman *et al.*, 2005). 7,8-Dihydroneopterin protects cells from hypochlorite by rapidly reacting with it (Gieseg *et al.*, 2001a, b). 7,8-Dihydroneopterin also appears to inactivate myeloperoxidase, the enzyme responsible for generating hypochlorite *in vivo* (Widner *et al.*, 2000; Razumovitch *et al.*, 2004).

## Pro-oxidant effects and cell signalling

With macrophages, neopterin has no apparent effect on cell survival whereas 7,8-dihydroneopterin protects HMDM and U37 cells from oxLDL and some oxidants (Gieseg *et al.*, 2002; Baird *et al.*, 2005). At the extremely high concentration of 5 mM, 7,8-dihydroneopterin causes sufficient oxidative stress to kill monocyte-like U937 cells (Baier-Bitterlich *et al.*, 1995), neuronal NT2/HNT cells (Spottl *et al.*, 2000) and the rat pheochromocytoma cell line PC12 (Enzinger *et al.*, 2002b) but only in the presence of TNF-α. Loss of mitochondrial dehydrogenase activity only occurs in ovarian carcinoma cell lines at 7,8-dihydroneopterin concentrations of 1 mM and higher (Rieder *et al.*, 2006). Likewise, with Jurkart T cells, 7,8-dihydroneopterin only induces apoptosis above 1 mM (Baier-Bitterlich *et al.*, 1996; Wirleitner *et al.*, 1998, 2001) via the redox-sensitive Bcl-2 pathway (Enzinger *et al.*, 2002a). All these mechanisms seem to involve the direct generation of oxidants within the tissue culture media due to the reducing activity of 7,8-dihydroneopterin as first observed in chemiluminescence assays (Oettl *et al.*, 1999). 7,8-Dihydroneopterin, like ascorbate (Buettner, 1988), may cause the reduction of redox-active metal ions within the buffers, which will increase the formation of various reactive oxygen species. This oxidant generation only becomes significant at extremely high millimolar 7,8-dihydroneopterin concentrations and in protein-free media. Proteins are very effective radical scavengers so the high concentration of soluble protein within plaque may make these reactions unlikely though still possible.

Neopterin had no effect on cell death in these experiments but did cause apoptosis at lower micromolar concentrations with vascular smooth muscle (Schobersterger *et al.*, 1996). The combination of interferon, TNF-α and between 10 and 100 μM neopterin caused iNOS activation and enhanced oxidative stress-triggering apoptosis (Hoffmann *et al.*, 1996, 1998). The effect was also seen with 100 μM 7,8-dihydroneopterin (Schobersterger *et al.*, 1996). This suggests that neopterin generated from oxidation of 7,8-dihydroneopterin during inflammation could trigger the death of the plaque smooth muscle cells, especially if there was sufficient levels of TNF-α.

Intracellular calcium in human-derived monocyte-like THP-1 cells is affected by micromolar levels of both neopterin and 7,8-dihydroneopterin and effectively inhibits ATP-induced calcium release from alveolar epithelial cells (Woll *et al.*, 1993; Hoffmann *et al.*, 2002). At similar concentrations, neopterin was also reported to cause cardiac contractile dysfunction in isolated perfused rat hearts (Margreiter *et al.*, 2000; Balogh *et al.*, 2005). The mechanism proposed behind this activity was oxidative stress but the reactive chemistry, and low oxidant yield observed with neopterin suggests a more direct mechanism in the cells and the intracellular calcium pools. The study clearly shows infusion of micromolar levels of pterin may have adverse clinical outcomes. The capacity of neopterin and 7,8-dihydroneopterin to cause calcium release may be important in plaque development where the formation of calcium deposits represents a serious deterioration in patient prognosis due to the increasing complexity of the plaque tissue.

## Atherosclerotic plaques

From the current literature, a hypothetical model can be drawn where interferon-stimulated macrophages release 7,8-dihydroneopterin to inhibit oxidation and cell death by scavenging oxidants generated by metal ions and superoxide released by cells. The 7,8-dihydroneopterin scavenges the neutrophil-released hypochlorite-producing neopterin, which inhibits further hypochlorite release via inhibition of myeloperoxidase. The neopterin also stimulates cell death in combination with TNF-α released by the various immune cells present. In experimental models of sepsis where healthy volunteers are infused with endotoxin, the peak in neopterin levels occurs 20 h after the peak in TNF-α (Fijen *et al.*, 2000) showing that 7,8-dihydroneopterin activity occurs late in the inflammatory process. But what happens during the chronic inflammatory process of atherosclerosis? Low levels of 7,8-dihydroneopterin and possibly 3HAA would shift the redox balance to oxidation causing oxLDL formation and cell necrosis/apoptosis. High hypochlorite formation would

generate elevated neopterin levels shutting off myeloperoxidase activity and possibly triggering neutrophil apoptosis. However, excess 7,8-dihydroneopterin synthesis with low levels of hypochlorite would give a high localized 7,8-dihydroneopterin concentration which would enhance the cell stability. Manipulation of this system could prevent the growth of plaques and their development to complex, unstable plaques through inhibiting oxLDL formation and cell apoptosis/necrosis.

The hypothesis that 7,8-dihydroneopterin is generated as an antioxidant also suggests a reason for the development of this response. The enzyme IDO which is also upregulated by $\gamma$-interferon is rapidly inhibited by nitric oxide (Thomas *et al.*, 1994). So it is possible that primate macrophages have evolved to suppress nitric oxide production by macrophages to preserve the activity of IDO. The result of this is that interferon stimulation of macrophages causes the synthesis of two potent antioxidants, 3HAA and 7,8-dihydroneopterin. This combination of antioxidants may allow primate macrophages to survive longer within sites of inflammation although this has yet to be shown. The down side of this mechanism may mean human macrophages survive longer within atherosclerotic plaques so enhancing plaque formation.

The key question is then, what is the *in vivo* concentration of neopterin and 7,8-dihydroneopterin? The *in vitro* studies clearly show that both 7,8-dihydroneopterin and neopterin could have significant effects on plaque growth. Our own studies on atherosclerotic plaques have shown that neopterin levels can be as high as 2.5 $\mu$M within some sections of the plaque (Figure 2). The labile nature of 7,8-dihydroneopterin makes it difficult to accurately measure 7,8-dihydroneopterin levels within plaques but it is possible that the concentration greatly exceeds the levels of neopterin measured. We feel it is likely that 7,8-dihydroneopterin/neopterin is being generated within plaques at concentrations that influence LDL oxidation and cell survival. The literature also suggests that where hypochlorite is oxidizing 7,8-dihydroneopterin to neopterin, the level of neopterin will be sufficiently high to promote cell death and plaque instability, especially where elevated levels of TNF-$\alpha$ occur. In support of this hypothesis, elevated levels of neopterin have been observed within unstable plaques (Garcia-Moll *et al.*, 2000a, b) and some correlation between plasma neopterin and TNF-$\alpha$ has been reported in atherosclerosis patients (Anwaar *et al.*, 1998). Also the lowering of neopterin levels with statin treatment is associated with increased patient survival (Neurauter *et al.*, 2003; Walter *et al.*, 2003). The control of the neopterin/7,8-dihydroneopterin system through specific anti-inflammatory agents may prevent the formation of complex plaques. $\gamma$-Interferon, GM-CSF and 1,25-dihydroxyvitamin D3 all increase 7,8-dihydroneopterin synthesis within macrophage cells (Schwende *et al.*, 1996) while histamine inhibits 7,8-dihydroneopterin synthesis (Gruber *et al.*, 2000). The difference in concentrations we have measured in plaques and those observed in tissue culture suggests there are other agents, which promote 7,8-dihydroneopterin/neopterin synthesis which may help control oxidative stress within plaques. The early observation that treatment of hepatitis C patients with $\gamma$-interferon decreased the level of serum lipid peroxides suggests that modulation of the 7,8-dihydroneopterin/neopterin system to the benefit of the patient might be possible (Higuras *et al.*, 1994). To achieve this, a greater understanding of the plaque concentration of 7,8-dihydroneopterin and how this relates to the other antioxidants and oxidants present is required. The role of neopterin and TNF-$\alpha$ induced death within plaques also needs to be further quantified.

## Conclusion

*In vitro* studies have demonstrated that neopterin and 7,8-dihydroneopterin could alter the redox balance within atherosclerotic plaques. 7,8-Dihydroneopterin promotes cell stability while inhibiting oxidative damage. Neopterin promotes cell apoptosis and changes in intracellular calcium. The balance between these two pterins *in vivo* is dependent on the rate of pterin synthesis in the macrophage and the level of hypochlorite and other oxidants within the plaque. Anti-inflammatory agents which promote 7,8-dihydroneopterin synthesis while inhibiting hypochlorite production may move the plaque oxidative environment to a less oxidative state so limiting oxLDL formation and cell death.

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## Conflicts of interest

The authors state no conflict of interest.

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